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**Potential Mechanisms Linking Nutrition and Neuroendocrine Control
of Reproduction in the Sheep**

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**Thesis submitted to the
School of Medicine at
West Virginia University
in partial fulfillment of the requirements
for the degree of**

**Master of Science
in
Physiology**

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ABSTRACT

Potential Mechanisms Linking Nutrition and Neuroendocrine Control of Reproduction in the Sheep

Adam B. Dobbins

Nutrition and reproduction are linked in that the nutritional status of an individual influences its reproductive performance, i.e. an individual in negative energy balance will have decreased ability to reproduce. The mechanisms in which nutrition influences reproduction are not understood. My data suggest that reductions in lutenizing hormone secretion resulting from photoperiod-induced increases in sensitivity to testosterone negative feedback may be mediated, at least in part, by elevated neuropeptide Y (NPY), a hypothalamic neuropeptide shown to alter gonadotropin releasing hormone release and potentially affect food intake. The results from my second study support our hypothesis that the growth hormone mediated hormone, insulin like growth factor-1 (IGF-1) increases thyroid hormone levels during food deprivation. Furthermore, results from these experiments show that food deprivation has a greater impact on males than females and that the thyroid hormone response to IGF-1 during food deprivation is much greater in females. Though there are likely many humoral and neuroendocrine factors that must act together to coordinate the reproductive response to nutrition, our studies with NPY and IGF-1 have begun to examine some potential mechanisms linking nutrition and neuroendocrine control of reproduction in the sheep

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CHAPTER 1: REVIEW OF LITERATURE

1) Seasonality in Female and Male Sheep

1.a.) Reproduction

1.a.1.) Reproductive Endocrine Axis

Control of reproductive function in mammals involves endocrine interactions among the hypothalamus, anterior pituitary, and gonads. Gonadotropin releasing hormone (GnRH) is the primary neurohormone regulating reproductive function. GnRH neurons, derived from the olfactory placode during development, are found spread throughout the preoptic area (POA) and medial basal hypothalamus (MBH) in sheep (1-4), as well as in rodents (5). These neurons are not located in any one particular nuclear region of the forebrain or hypothalamus. Among various species 40-50% of GnRH neurons are thought to terminate in the median eminence (1;3-8). With regard to the sheep, the majority of GnRH neurons are found in the medial POA with a significantly greater number of neurons projecting from here to the median eminence than from other areas, indicating that the POA likely gives rise to the majority of GnRH that is released (3). GnRH is released in a pulsatile fashion into hypothalamo-pituitary portal blood leading to the pulsatile release of luteinizing hormone (LH), and a relatively continuous release of follicle stimulating hormone (FSH), from the anterior pituitary gland. Both LH and FSH are involved in regulating production of gametes and hormones in males and females. The frequency of GnRH pulses, and consequently LH pulses, is the code used by the nervous system to control testicular/ovarian function (9). Due to its rapid breakdown in the peripheral vasculature, GnRH is only measurable in the portal blood, and is therefore very difficult to assess. Thus, LH is often used as a peripheral marker to

reflect the activity of a neural oscillator, or pulse generator, that produces episodic secretions of GnRH. At least in the sheep, each LH pulse is preceded by a GnRH pulse (10).

Release of GnRH and LH/FSH is impacted greatly by circulating gonadal steroids. Testosterone is predominantly released by the testes and can be converted into dihydrotestosterone (DHT) via the enzyme 5- α -reductase, or into estradiol via the enzyme aromatase. DHT and estradiol feed back at the hypothalamus to decrease GnRH release and at the pituitary to decrease the response to GnRH. In the female, LH and FSH stimulate ovarian production of estradiol and progesterone. Progesterone and low levels of estradiol act to inhibit GnRH release and alter pituitary sensitivity to GnRH. However, high levels of estradiol cause the pre-ovulatory GnRH and LH surge. Sensitivity to steroid negative feedback changes throughout the year, so that GnRH and LH secretion is relatively high during the breeding season and decreased during the non-breeding season.

1.a.2.) Seasonal changes in GnRH/LH Release

Sheep are seasonal breeders with initiation of reproductive activity occurring during the shortening days of late summer and fall, and terminating during the lengthening days of late winter or early spring (11;12). These seasonal swings in reproductive activity are due largely to changes in pulsatile patterns of GnRH, and thus LH, secretion. The alterations in patterns of GnRH and LH release are due to an interaction of daylength and steroid negative feedback (13-17). Based on the expression of estrous cycles, female sheep demonstrate an abrupt change from breeding season to non-breeding season. In ewes that have been ovariectomized and implanted with

estradiol, GnRH and LH secretion exhibit a high frequency pattern of release during the breeding season, whereas during the nonbreeding season, GnRH and LH secretion is characterized by a low-frequency pattern of release. Within the breeding season of ewes, pulses of LH and GnRH are infrequent during the luteal phase of the estrous cycle, reported at 0-2 pulses over a 6-hour period (18). This is due to the presence of a progesterone-producing corpus luteum at the site of ovarian rupture. During pregnancy, high progesterone acts as an inhibitor of LH secretion (19;20), inhibiting estrous cycles and allowing the individual to maintain the pregnancy. A low level of estradiol is maintained throughout the luteal phase, which acts to enhance the inhibitory effects of progesterone; estradiol reduces pulse amplitude, while progesterone reduces pulse frequency (21). During the follicular phase, GnRH and LH pulse frequency increases dramatically to 8 pulses per 6 hour period following withdrawal of progesterone during luteolysis (18). During this period, estradiol is a weak inhibitor of LH release and does not suppress LH pulse frequency (13), but rather increases pulse frequency (22). During the anestrous period, however, estradiol is an extremely powerful inhibitor of LH pulse frequency (23;24) and contributes to decreased pulsatile GnRH and LH release (25), which drops to a frequency of 1-2 pulses per 12 hour period (18). These changes in estradiol negative feedback are, in large part, responsible for seasonal variations in LH release.

Males also show seasonal alterations in fertility, though changes among seasons are more gradual than in females. Lincoln (26) reviewed the seasonal changes in reproduction in males of the Soay breed of sheep. There is a seasonal cycle in the blood concentrations of FSH with a marked increase from June to September, consistent with

its role in stimulating spermatogenesis and growth of the testis. The testes are fully enlarged in October at the beginning of the rut, when most matings occur in the wild. In other, less seasonal breeds, like Hampshire and Suffolk, no seasonal change in LH is reported (27;28). Furthermore, seasonal changes do not occur in the same time frame for males and females. LH concentrations rise earlier in the fall in adult males than in adult females (29), possibly due to differing sensitivity to estradiol and testosterone between the two sexes. Nonetheless, comparable circannual changes in gonadal feedback exist in the male as in the female. Entry into the breeding season is governed by an increase in pulsatile LH secretion as a result of a reduced response to testosterone negative feedback in rams. Testosterone is converted into estradiol via aromatization and into DHT via 5- α -reduction and the relative contribution of each of these metabolites for controlling GnRH release is unclear. Estradiol is inhibitory to LH at physiological concentrations in steers (30) as well as sheep (31;32) and blockade of the conversion of testosterone to estradiol increases LH secretion in male sheep (33). Castrated rams given DHT demonstrated a lack of responsiveness of LH release to exogenous GnRH when compared to untreated animals (34). DHT appears to be of physiological importance since blocking 5- α -reductase activity significantly blunts the ability of testosterone to inhibit pulsatile LH release (35), possibly acting at the pituitary since there is a large amount of 5- α -reductase activity (36) and a high number of DHT binding sites (37) in male and female calf pituitary glands.

Photoperiod provides input to control the timing of changes in steroid sensitivity. A switch from long to short days results in a progressive decline in LH concentrations, while a switch back to long days caused the opposite response in LH (17). These

observations lead to the conclusion that the inhibitory photoperiod of anestrous enables estradiol and/or DHT to act within the brain to depress LH pulse frequency. This action is significantly diminished by the inductive photoperiods of the breeding season (38).

1.a.3.) Mechanisms of Steroid Negative Feedback

The neural mechanisms that mediate steroid negative feedback on gonadotropin release are not fully understood. In male sheep, testosterone, or its metabolites like DHT and estradiol, acts primarily, although not exclusively, on the brain to suppress GnRH pulse frequency. Specifically, castration leads to an increase in GnRH pulse frequency, yet LH pulse frequency does not increase significantly (39), whereas testosterone replacement reduces GnRH pulse frequency, yet has a marginal effect on pituitary response to exogenous GnRH (40). Evaluation of pituitary responsiveness across various times of the year leads to the conclusion that testosterone does act directly on the pituitary to modulate the response to GnRH, yet since LH pulses still mirror GnRH pulses, it is still maintained that shifts in LH pulse frequency reflect parallel changes in GnRH pulse frequency rather than profound changes in pituitary responsiveness to GnRH (27). The neural sensitivity to the negative feedback action of testosterone as well as circulating concentrations of testosterone vary greatly with stage of the annual reproductive cycle (27). Thus it is likely that the relative effect of testosterone on the pituitary versus brain also varies with stage of the annual reproductive cycle (41).

Cells containing estrogen receptors (ER) in the brain of the ewe are distributed throughout many areas of the brain and hypothalamus, including the medial POA, anterior hypothalamic area, ventromedial septum, bed nucleus of the stria terminals (BNST), ventral medial nucleus (VMN), and arcuate nucleus (Arc) (42-44). Similar to

guinea pigs, but not to rats or monkeys (45), ER-immunoreactive cells have been detected in the supraoptic nucleus (SON) of ewes within its dorsomedial aspect (43). Only very few ER-immunoreactive cells are found in the paraventricular nucleus (PVN). In ewes, the highest density of ER-immunoreactive cells is found in the Arc (42), and ER have been reported in the anterior pituitary gland (46-48). Blache *et al.* (42) used brain sections from both ovariectomized and estradiol-replaced ovariectomized ewes and found no effect of estradiol on ER-positive cell density in the POA, VMN, or Arc. However, progesterone administration did result in the detection of greater numbers of ER-immunopositive cells in the VMN of ovariectomized ewes (42). In the VMN of male sheep there are substantially fewer ER α containing cells and less ER α mRNA per cell than ewes (49). In addition, a similar finding for the mPOA and VMN has been reported for rats (50). The more recently discovered estrogen receptor- β (ER β) is present in the Arc, VMN, POA, SON, and PVN within the hypothalamus (49;51), though its role in mediating various aspects of the GnRH response to estradiol remains unclear (52).

In general, the overall distribution of androgen receptor (AR)-containing cells in males is very similar to that of ER in ewes, with the largest numbers of AR-immunopositive cells found in the medial POA, VMN, and Arc (45). In the VMN, AR-positive cells are located more medially in the dorsomedial division whereas estrogen receptors are not found in this particular subregion (45). AR-positive cells are detected throughout the Arc, but at a much lower density compared to ER-containing cells in ewes (45). A very similar distribution has been shown for AR-containing cells in rat hypothalami (53).

GnRH neurons in female and male rats do not possess ER α (54;55). Studies with ovariectomized ewes have similarly demonstrated that GnRH neurons throughout the hypothalamus do not display ER immunoreactivity (43;56). Likewise, GnRH neurons of rats do not possess AR (57). A consistent observation from these double-labeling studies is that GnRH neurons are usually surrounded by ER α and AR-immunopositive cells (45;55;56). Based on these studies, it seems likely therefore that steroid-receptive interneuron populations mediate the influences of estradiol and androgens (i.e. testosterone and DHT) upon GnRH neurons.

In the absence of estradiol in the female sheep (58), or in the absence of testosterone in the male sheep (17;59), a relatively small photoperiod-induced decline in LH pulse frequency and an increase in pulse amplitude still occurs when animals are exposed to natural or artificial long-day photoperiods. Larger steroid-independent suppression of circulating gonadotropin concentrations during inhibitory photoperiods are apparent in other species such as red deer hinds (60), and snowshoe hare bucks and does (61). These steroid-independent rhythms are often considered to be the result of variations in an underlying direct photoperiodic drive to gonadotropic output (58;61), which is further modified by steroid negative feedback (38). Lincoln (17) reported similar results for rams under controlled, artificial lighting. Other hormones, such as thyroid hormones (62), may play a role in these steroid independent changes in LH.

1.b) Food Intake

1.b.1.) Influence of Photoperiod

Many species living in temperate and cold climates express profound seasonal changes in voluntary food intake (VFI) and body weight. There is evidence that long-

term cycles in VFI, growth, and pituitary hormone secretion persist in sheep living under constant photoperiod and environmental conditions (63). This supports the view that the seasonal cycle in VFI is generated endogenously. Under natural conditions, the response to the annual cycle in day length acts to entrain the period of endogenous rhythm to a period of exactly 12 months in order to time the anabolic growth phase to summer when food is most abundant and the catabolic anorexic phase to winter when food is scarce (64;65). Artificial conversion from short days to long days provokes an increase in VFI and exposure to alternating 3-6 month periods of long and short days drives the cycle in VFI and body weight (65). These photoperiod-induced changes are controlled, in large part, by mechanisms residing within the hypothalamus.

Many neuropeptides that have been isolated in the hypothalamus are capable of influencing VFI. Orexigenic peptides, such as neuropeptide Y (NPY) and agouti-related peptide (AgRP), are up-regulated under long days in sheep (65) and hamsters (66) in association with an increase in appetite in males. An increase in arcuate NPY has been reported in females on a long day photoperiod (64). Barker-Gibb *et al.* (67) reported that seasonal changes in NPY in the ewe are not dependent on steroids. In the male, expression of the proopiomelanocortin (POMC) gene is reduced under long days in testosterone-implanted castrate sheep (68) and hamsters (66), while being increased during long days in ovariectomized ewes (64). One potential reason for these differences could be due to gender. Alternatively, NPY and POMC may be regulated differently in the context of season and steroid milieu. Orexigenic melanin concentrating hormone (MCH) is reduced under long days in males, but changes in expression may be linked to its role in regulating the reproductive axis more so than regulating VFI (65). It is

important to note that POMC serves as a precursor to both anorexic α -melanocyte-stimulating hormone (α MSH) and orexigenic β -endorphin so its role in controlling food intake is more complicated (69).

1.b.2.) Role of Steroids

Gonadal hormones are involved in the regulating food intake in rats (70;71), hamsters (72), and mice (73). Androgens generally act as anabolic agents that increase food intake and lean mass, whereas estrogens are catabolic and decrease food intake and body weight. Male rats that are orchietomized at birth exhibit significant decreases in rate of body weight gain with a slight or no decrease in daily food intake and testosterone administration reverses the effect on body weight (70;73-82). Orchietomy has no detectable effects on the quantity of food ingested in adult rats until after about 4 weeks of age, by which time lean body mass has also decreased (83). Low dose androgen administration increases food intake in rats, whereas higher doses decrease food intake (83). Treatment with androsta-1,4,6-triene-3,17-dione, an aromatase inhibitor, increased food intake and weight gain when administered with high doses of testosterone (84). This supports the idea that dose dependent effects of testosterone result from the aromatization to estrogens when testosterone is given in high doses.

In the female, estradiol significantly influences energy balance by reducing food intake. Ovariectomy produces an immediate increase in feeding in rats and mice, and estradiol treatment is sufficient to normalize both feeding and body weight (85-88). In postmenopausal women, there is an increase in total percent body fat. Hormone replacement attenuates these fat gains (89). During estrous, ovariectomy eliminates an estrous-induced decrease in food intake (90). Several studies have reported an inhibitory

effect of estradiol on NPY mRNA or peptide levels in female rats (91-94). Decreasing NPY input could be one mechanism by which estradiol helps to decrease food intake.

NPY levels are higher in males than in females. Sexually dimorphic NPY levels may, at least in part, be due to the metabolism of testosterone to dihydrotestosterone (DHT). While the influence of DHT on NPY has not been directly assessed, like testosterone, DHT prevents cold-induced loss of body weight (95), increases body mass (76), and increases food intake (70;96). This raises the possibility that DHT may mediate, at least in part, metabolic responses involving NPY that are influenced by testosterone. Indirect evidence also indicates that in human subjects some of the effects of gonadal steroids on food intake and body weight may be mediated to some extent by influencing serum leptin levels and hypothalamic leptin receptor gene expression (97).

2) Influence of Nutrition on Reproduction

Producers have long known the importance of proper nutrition for increasing conception rates and decreasing postpartum intervals. The mechanisms whereby nutrition influences reproduction are not completely understood. In the following section, the influences of nutrition on three hallmarks of reproduction (puberty, postpartum return to breeding, and expression of estrous cycles) will be discussed, along with potential mechanisms that link nutrition and GnRH/LH secretion.

2.a.) Nutritional Influences on Puberty, Postpartum Interval, and Estrous Cycle Expression

Puberty is the time in life when mature gametes are first produced and individuals become capable of reproducing. This is due to an increase in activity from the

reproductive endocrine axis, i.e. an increase in GnRH and LH release. In the sheep this is the result of a decreased responsiveness of the hypothalamus to estradiol negative feedback. As measured by an increase in LH, puberty occurs earlier in the male lamb than in the ovariectomized female lamb. In the immature female, LH pulse frequency is low and increases typically at 25-35 weeks in spring born lambs (98). LH pulse frequency is also initially low in the male, but increases to near pubertal levels by 9-11 weeks (99). This sex difference is due to an earlier decrease in response to steroid negative feedback in the males (100). Foster *et al.* (101) failed to find an increase in the frequency of pulsatile LH secretion in ovariectomized lambs at the time when intact lambs began their first reproductive cycles suggesting that steroid-independent mechanisms are likely not overly important. They also ruled out a major influence of photoperiod in ovariectomized lambs as LH pulse frequency was not influenced by a photoperiod treatment known to delay puberty in gonad-intact sheep. However, insufficient nutrition can delay the peripubertal rise in pulsatile LH secretion that normally occurs during the stimulatory daylengths of autumn (102). Still, photoperiod may play a modulatory role in the onset of puberty. In males, a pre-pubertal increase in gonadotropins drives increasing testosterone and spermatogenesis to achieve a level of fertility approximating that of the adult male during the nonbreeding season (103). Once testicular function approaches a level similar to that of the seasonally suppressed adult male, subsequent sexual maturation to reach full fertility is more photoperiod-dependent (104).

In other species, a change in hypothalamic sensitivity to estradiol negative feedback does not seem to be a component of puberty onset. Ojeda *et al.* (105) has

reviewed the control of puberty in the female rat. In this model the CNS appears to play a pivotal role. In the time leading up to puberty, there is a centrally driven, gonadal-independent diurnal change in pulsatile LH release. Basal release of LH and the magnitude of LH secretory episodes increase markedly in the afternoon. These changes stimulate the ovary to produce more estradiol which then evokes minisurges of LH secretion that in turn further stimulate ovarian development and steroidogenesis. The increase in ovarian steroids, especially estradiol, helps lead to the preovulatory discharge of GnRH which directly promotes the first surge of gonadotropins and ovulation.

As with sheep, GnRH neurons in primates appear to be functionally mature well before the onset of puberty in primates (106;107). In fact, following gonadectomy at 1-2 weeks of age, there is up to 10 weeks of elevated mean LH levels observed in both the male and female monkey, though the increase is more dramatic in the male (108). The postnatal increase in LH release ends due to central inhibition rather than due to steroid negative feedback (109;110). Richter and Terasawa (111) reviewed the mechanisms that are responsible for maintaining gonadotropins at a low level before puberty in the rhesus monkey, and how these inhibitory mechanisms are diminished at puberty. Gamma-aminobutyric acid (GABA), an inhibitory neurotransmitter, along with possible involvement from additional factors such as NPY, are involved in inhibiting GnRH release from the hypothalamus. At the onset of puberty in the rhesus monkey there is a postulated decrease in inhibitory GABA input and an increase in levels of the excitatory neurotransmitter glutamate, which allows for an increase in LH pulse frequency and the initiation of estrous. NPY and its role in puberty in primates appears to be sex specific. In the female rhesus monkey there is an increase in NPY mRNA along with the increase

in GnRH, indicating NPY may be stimulatory to GnRH (111). In the male there is a decrease in NPY mRNA along with an increase in GnRH mRNA suggesting that NPY is inhibiting GnRH (112).

Regardless of the strategy whereby puberty is attained, onset of puberty is delayed in all species whenever a female is in negative energy balance (113). Furthermore, the onset of fertility is also more closely associated with body size and food intake than with chronological age in females whose growth has been retarded by food restriction (114). Foster and Olster (102) demonstrated that ewe lambs fed to maintain their weaning weights were anovulatory at a time when growing lambs became pubertal. McShane and Keisler (115) demonstrated that hourly injections of LH in underfed lambs induced follicular growth, LH surges of normal amplitude, and ovulation, indicating that the hypothalamus is likely involved in decreased reproductive function during times of reduced nutrition. Indeed, GnRH secretion is inhibited in undernourished lambs (116). These studies suggest that undernutrition acts to inhibit the increase in GnRH secretion that is required for puberty to occur. LH pulses and LH surges have been induced in peripubertal female lambs by administration of GnRH (117), showing that the pituitary and the ovary are not limiting in pubertal development. GnRH neurons also seem mature prior to puberty as evidenced by stimulation of LH release using N-methyl-D-aspartate, a glutamate analog that triggers GnRH release (117). Refeeding is known to increase circulating levels of gonadotropins and trigger onset of puberty and expression of estrous cycles in growth-restricted animals, and exogenous administration of GnRH pulses to growth-restricted prepubertal rats will induce estrous cycles (118).

Like puberty, the duration of the postpartum interval, or the time period following parturition when reproduction is inhibited, is also influenced by nutrition. Postpartum anestrus is a transition period during which the functional hypothalamic-pituitary-ovarian-uterine axis recovers from the previous pregnancy. Time is necessary for uterine involution to begin, for anterior pituitary LH stores to be replenished, and for waves of ovarian follicular growth to resume (119). During pregnancy, high circulating estradiol concentrations inhibit the pulsatile release of GnRH from the hypothalamus. The dramatic fall in circulating estradiol concentrations at the time of parturition leads to an immediate postpartum increase in FSH secretion, which initiates a wave of ovarian follicular growth within two weeks postpartum in cattle (120) and in sheep (121). However, follicle maturation and ovulation do not occur unless there is a concomitant increase in LH pulsatility.

Duration of postpartum anestrus is affected by four major factors: suckling, age, season, and nutrition (119). Suckling increases the duration of postpartum anestrus and old or first pregnancy animals often rebreed more slowly. Restriction of dietary energy late in the prepartum period or early in the postpartum period reduces the number of animals returning to estrus within a well-defined breeding season. These effects are apparently attributed to a failure to develop preovulatory ovarian follicles (122). Most studies examining the postpartum interval have been conducted in cattle. Increasing energy intake after parturition in thin heifers shortened the postpartum interval (123) and increased circulating LH concentrations (124). Body condition score at parturition is positively correlated with follicular development early post partum (125) and pituitary LH content at 30 days postpartum (126).

Most ewes give birth at the end of their natural breeding season when photoperiod is inhibitory to the reproductive axis, making the postpartum period less of a concern in this species. Nonetheless, a limited body of literature exists concerning postpartum intervals in sheep. Autumn lambing ewes maintain the opportunity to re-breed during the fall breeding season. Economically this could be beneficial if a second lamb crop can be produced in one year, or if the producer has a high priced niche market during the holiday season. Mandiki *et al.* (121) have shown November lambing ewes have a shorter postpartum interval than July lambing ewes. Lishman *et al.* (127) did report that restricting food intake for 84 days postpartum in ewes that lambed in the autumn reduced the percentage of ewes returning to estrous. Since dietary energy restriction impairs pulsatile LH secretion in ovariectomized ewes, it seems reasonable to suggest that energy restriction would delay or prevent postpartum re-breeding of ewes via alterations in GnRH/LH release.

Similar to puberty and postpartum return to breeding, inadequate nutrition also inhibits the expression of estrous cycles. For example, severely food deprived rats have lower pituitary, ovarian, and uterine weights (128). Restricted feeding for a prolonged period affects ovulation rate, timing of the estrous cycle, and ability to reproduce in rodents (128;129). Tropp and Markus (130) have shown that in Fischer 344 and Brown Norway rats moderate food restriction (to produce a 15% drop in body weight) can cause cessation of estrous cycles as early as day one following the onset of food deprivation. Ad libitum feeding restored estrous cycles in these animals within 2-4 days. In this study, Sprague-Dawley and Long-Evans rats were more resistant to the effects of mild

food restriction than Fischer 344 and Brown Norway rats, indicating that, in rodents, strain-dependent differences in response to food restriction exist.

Restricted feeding and acute fasting in heifers inhibit cyclic ovarian function and expression of estrous, and prolonged restriction of dietary energy results in cessation of estrous cycles in cattle (131;132). Failure to ovulate occurred 32 weeks after initiation of food restriction to cause a 1% loss of body weight per week (22% average weight loss over treatment period was actually achieved) (133). Anovulation was likely due to the observed significant drop in LH and FSH levels. In addition, circulating IGF-1, insulin and glucose levels were reduced in restricted animals. The potential role of these factors in mediating the influences of nutrition on reproduction will be discussed later.

Adult ewes maintained on low planes of nutrition also exhibit reduced ovulation rates (134), a response attributed to reduced ovarian follicular development (135) rather than to alteration of the pre-ovulatory surge of LH. Effects on follicular development could result from disruption of pulsatile LH release, and indeed ovariectomized ewes maintained on a low energy diet exhibit fewer LH pulses and lower mean LH concentrations in serum (136).

Flushing, or increasing energy intake at least one estrous cycle prior to breeding, in ewes with low body condition is one way of increasing conception rates and the number of offspring conceived (134). Ovulation rate in the ewe is influenced by both body condition at mating and the pre-mating level of nutrition (137;138). These two phenomena are clearly related since long-term changes in pre-mating level of nutrition will change the body condition of the ewe at mating (134). The number of eggs ovulated,

and ultimately the number of lambs born, can be increased in underconditioned ewes by flushing (139).

2.b.) Site of Influence

As discussed above, although GnRH release is inhibited during undernutrition, GnRH neurons themselves appear to be functionally normal during undernutrition. The finding that pulsatile LH secretion can be suppressed in gonadectomized animals suggests that food restriction can act independently of steroids to suppress GnRH release. However, evidence exists to suggest that food restriction enhances the ability of estradiol to inhibit pulsatile LH secretion. The systems underlying this response are unknown. A number of neural systems have been shown to express ER α and/or AR and also influence GnRH release. Such potential mediators of steroid action on GnRH release include galanin (140;141), classical neurotransmitters such as serotonin (142), excitatory (143) and inhibitory amino acids (144), and local growth factors (145). Some of these neural mediators of steroid feedback are listed in Table 1 at the end of chapter, along with their action on GnRH and LH secretion.

NPY is one particularly attractive candidate that may be involved in mediating steroid negative feedback on GnRH release during undernutrition. NPY, steroid negative feedback and GnRH release during inadequate food intake could be linked for the following reasons: NPY alters GnRH and LH release (67;146-148), NPY-containing neurons synapse directly onto GnRH neurons (149), NPY neurons coexpress ER α (150), and NPY levels are markedly influenced by nutrition (151). This neurotransmitter will be discussed in greater detail later in this section.

2.c.) Male-Female Differences

There are several differences between males and females in the way they respond to changes in nutrition. Hoyenga and Hoyenga (152) have proposed that due to gender specific evolutionary selection pressures, female mammals have evolved a greater capacity to conserve energy than males. Accordingly, food restriction has been reported to have a greater and more permanent effect on physical growth in males than females (152). In rats subjected to complete starvation for six days, body mass loss was similar following starvation, but males lost a greater percentage (16%) of their body mass as protein than did females (8%). The ratio of lipid to protein lost (g) was 1.16 for males versus 3.26 for females (153). Taken together these data suggest that females are more adept at conserving energy during periods of low fuel availability. Differences are also observed when animals have access to excess calories. Hileman *et al.* (154) show that male mice fed a high fat diet gained weight much more rapidly than did female mice, with increased weight gain being evident by 4 weeks in males. Females, however, did not show appreciable additional weight gain until week 8, and this was largely due to an increased response of one female to the high fat diet. Females appeared to be more able to compensate by decreasing intake, either in total grams of food ingested or kilocalories eaten, than were the males on a high fat diet.

Several factors that are involved in regulating body weight show sexual dimorphism. There is a clear effect of sex and season on the satiety functions of leptin in sheep. During the shortening days of fall, leptin was ineffective as a satiety agent in either sex. During the lengthening days of spring, however leptin was able to suppress appetite to a greater degree in female than in male sheep (69). There is also a gender

difference in the development of leptin resistance in mice following exposure to a high-fat diet. Males placed on a high-fat diet for 5 weeks did not lose body fat in response to physiological doses of leptin infused peripherally. In contrast, female mice on the same treatment, as well as males and females fed the high-fat diet for 15 weeks, lost body fat during the leptin infusions (155). There are conflicting data concerning leptin in males and females. In rats and mice, leptin levels have been reported to be higher in either males (156) or females (97;156;157). In humans, females consistently have higher leptin levels than males (158). There is less of a decrease in leptin due to starvation in females compared to males (157), though the mechanisms underlying these sex-related differences remain unclear.

In response to fasting, there is a greater reduction in total T_4 in females than in males, though both sexes showed a decrease in T_4 following starvation (159). A reduction in T_4 is consistent with previous studies in both males (160) and females (161) although direct comparisons between sexes were not performed. Corticosterone levels following fasting increased to a greater extent for females than for males (159), possibly due to a more robust metabolic response to food deprivation in females. It is also possible that starvation may be more stressful for females. Sex-related differences in the response to stress have been reported (162), and Born *et al.* (163) have proposed that, at least in humans, young females are more sensitive to corticosterone releasing hormone (CRH) since higher CRH levels were required to elicit the same corticosterone levels in young males. The magnitude of neuroendocrine and metabolic responses to hypoglycemia is reduced in women compared with men (164;165). These data suggest that females are less insulin sensitive compared to males, at least under hypoglycemic

conditions (165). Circulating IGF-1 concentrations were greater in prepubertal male sheep than prepubertal female sheep (166;167). Accordingly, greater concentrations of plasma IGF-1 were found in rams than in ewes around the time of puberty (168), but in this study sex differences before puberty were not detected.

As mentioned earlier, a sex-dependent difference in the timing of puberty exists for male and female sheep. These differences arise because the time course of gametogenesis and the metabolic investments in reproduction can be quite different in the two sexes. It takes longer for gametogenesis in males so there must be a time difference in order for them to be prepared to breed at the same time as the females. As mentioned earlier, the pubertal increase in LH secretion occurs in male sheep at about 10 weeks and in female sheep at about 30 weeks of age (169). This is due to a sex-dependent difference in responsiveness to inhibition of GnRH secretion by estradiol. Thus, the period of high responsiveness to estradiol lasts three-fold longer in prepubertal female sheep than in male sheep (100). The stimulatory feedback mechanism by which estradiol induces LH surges is also sexually differentiated in sheep (32). In castrated adult sheep, treatment with high physiological levels of estradiol promptly suppresses tonic LH secretion in both sexes. However, within 24 hours this negative feedback response is followed by an LH surge only in females. With regard to seasonal fertility, reproductive activity is highly seasonal in the female sheep, and tends to be all or none, whereas the male remains fertile year-round with relatively less seasonal variation in fecundity and libido (26). Seasonal changes in LH levels do not occur in the same time frame between males and females as LH concentrations rise earlier in the fall in adult males than in adult

females (29), possibly due to differing influences of estradiol and testosterone between the two sexes.

2.d.) Theories concerning the link between nutrition and reproduction

The reduction in GnRH and LH pulse frequency observed during dietary energy restriction is dramatic and probably represents the most important means by which undernutrition impairs reproductive activity. Early observations in humans that menarche occurred at a critical level of body fatness led to the hypothesis that body fatness somehow regulates reproductive activity (170). The critical body fat hypothesis is supported by data from a wide variety of species and physiological situations. Body weight is related to mean LH levels and the continued expression of estrous cycles in cattle (132;171) and is correlated with the postpartum interval (172). Because weight loss in adult animals is primarily due to a loss of fat, the reduction in LH pulse frequency associated with prolonged dietary energy restriction or decrease in bodyweight/body condition scores is associated with a reduction in body fatness (132;136). The extent of an animal's energy reserve can also influence the time required for undernutrition to alter the reproductive axis. A two-day fast blocks estrous cycles in lean, but not fat hamsters (173). Thus, it seems plausible that metabolic changes resulting from changes in nutrition and paralleling changes in body adiposity somehow regulate pulsatile LH release.

Body fat could exert its effects on reproduction through an alteration in steroid metabolism (174). Changes in body weight and body fat are associated with changes in estradiol metabolism (174) and a high level of androgen aromatization into estradiol occurs in adipose tissue (175). However, impaired tonic LH secretion caused by

undernutrition is observed in gonadectomized lambs (102), rats (176), and the rhesus monkey (177). If body fatness does alter the activity of the reproductive axis, it is probably through steroid-independent or steroid-modulated mechanisms.

Arguing against a direct role for body fat as a trigger for reproduction are a number of experiments having demonstrated that body fat lacks a direct physiological or biological relationship to the onset of estrous cycles (178). In intensively trained female athletes, menarche is not achieved for months following the attainment of a body weight and composition expected to produce menarche in girls not subjected to such intense training (179). In addition, in some studies puberty and body adiposity can be dissociated (180-183). In addition, when metabolic fuels are supplied, the level of body fat is irrelevant to the maintenance of estrous cycles (173). Instead, the amount of energy stored in adipose tissue could be an important component of overall energy balance, and body fat is probably a marker for the availability of energy for reproductive activity.

The central nervous system still must have some way of detecting changes in the state of an individual's energy balance in order to effect changes in GnRH/LH secretion. Some signal(s) must exist to trigger central changes in response to the body's energy state. Blood-borne signals such as metabolites, metabolic fuels, or hormones may serve as a signal to the brain indicating the current energy state of the organism. This signal must have the following characteristics: 1) must be secreted in proportion to body energy status, 2) must have access to the brain, and 3) must effect a predictable response. The following section will examine some possible metabolic and humoral cues that may serve as signals to the central nervous system.

3) Potential Metabolic Signals linking Reproduction and Nutrition

3.a.) Insulin-like growth factor 1 (IGF-1)

Growth is regulated by a number of factors, including environmental signals, health, nutrition, and the genetic potential for growth of an individual. The IGF's are endocrine signals that are critical for normal growth. IGF-1 and IGF-II were first isolated in large-scale purification from a human plasma fraction by Rinderknecht and Humbel (184). They are single chain polypeptides with structural homology to proinsulin. They regulate proliferation and differentiation of a multitude of cell types and are capable of exerting insulin-like metabolic effects. Traditionally, IGF-II is considered to act in this manner pre-natally and IGF-1 to act in young and adult animals. They are produced by most tissues of the body (185-187), are abundant in the circulation, and act in autocrine, paracrine, and endocrine fashions (188). IGF-1 activity is regulated in part by association with binding proteins. IGF-1 and IGF-binding proteins (IGFBPs) are present in the anterior pituitary gland and hypothalamus (189), as well as serum and liver in ruminants (190).

IGF-1 is part of the growth hormone (GH) axis, where GH-Releasing Hormone and Somatostatin are released from the hypothalamus to stimulate and inhibit, respectively, the secretion of GH from the pituitary gland. IGF-1 production by the liver is stimulated by pituitary GH (191) and accounts for about 75% of circulating IGF-1 levels (192). Circulating IGF-1 feeds back directly on the anterior pituitary to inhibit the synthesis and release of GH (185;193-195).

3.a.1.) IGF-1 and Growth/Nutrition

IGF-II is classically thought of as the primary IGF involved in prenatal development. IGF-II is expressed more widely in the embryo (196) and is required for normal embryological development, as IGF-II null mice are 60% smaller than normal littermates (197;198). However, IGF-1 is also essential for embryological development. IGF-1 knockout mice were not only 60% smaller than littermates, but displayed severe muscle dystrophy and a high percentage died at birth (199).

Postnatal growth is dependent on normal pulsatile secretion of GH from pituitary somatotrophs. This is particularly important for the accelerated growth seen during the peri-pubertal period. Loss of GH secretion can have important deleterious effects in the maturing organism. GH deficiency results in growth retardation as pituitary-deficient rats did not gain weight or grow in length unless administered GH or IGF-1 (200). In addition to increasing bone length and width and skeletal muscle growth, GH and IGF-1 increase kidney, spleen, and thymus weight, and decrease the weight of epididymal fat pads (201). Despite having markedly increased circulating GH levels, IGF-1 knockout mice are small, suggesting that the major effects of GH on growth are dependent on IGF-1 expression (192). In a study of GH deficient rats, administration of human GH (hGH) had no significant effect on relative weights of various organs, whereas a high dose of hIGF-1 had significant stimulatory effects on the kidney and spleen. Both hGH and hIGF-1 increased the relative weight of the adrenals, but this was only significant for hIGF-1 treatment (200).

IGF-1 levels are affected by a lack of dietary energy. Food restriction decreases serum IGF-1 in rodents (202;203), lactating rats (204), ruminants (205), and humans

(206;207). Furthermore decreased IGF-1 due to food restriction cannot be reversed by administration of GH (206). In guinea pigs, IGF-1 was significantly reduced in animals fed 70% of their normal diet for a period of 3-4 days (203). There was a significant IGF-1 response to a 24-hr food restriction at 5 weeks of age in mouse lines that were bred both for lean body mass increase and fat body mass increase (202). Across other species there is a delay in IGF-1 response to nutrition deficiency. In adult canines it takes 4 days of fasting before a decrease in IGF-1 is seen (208), and in adult humans there is also a decrease in IGF-1 over 9 days of food restriction (206). The decline in IGF-1 concentrations during fasting is due to a decrease in binding of GH to its hepatic receptor, even though GH levels remain constant or even increase. These changes are accompanied by decreases in hepatic IGF-1 and GH receptor mRNA abundance (209;210). Thus, during food restriction, the ability of GH to induce IGF-1 is reduced.

3.a.2. IGF-1 and Reproduction

Several studies have implicated IGF-1 as a putative metabolic signal linking nutritional state with reproduction. The ability of IGF-1 to act directly on the hypothalamus of immature animals to affect GnRH neuronal activity was first shown *in vitro* when IGF-1 stimulated GnRH release from the rat median eminence (211). Other *in vitro* experiments have shown that IGF-1 can have an inhibitory effect on the release of GnRH (212). These researchers applied IGF-1 to a larger hypothalamic area, and postulate that differences may be due to site-specific differences in IGF-1 inputs controlling GnRH secretion. *In vivo*, female rats exhibited an increase in LH release following i.c.v. administration of IGF-1, apparently due to an increase in GnRH as the response was blocked by administration of antibodies to GnRH (213).

IGF-1 may play a role in puberty onset and appears to act at all levels of the HPG axis to influence reproduction. IGF-1 crosses the blood-brain barrier by a specific transport system and is present in the CSF (214). IGF-1 receptors are widely distributed in the hypothalamus and even more abundantly expressed in the pituitary (215). Locally produced IGF-1 augments ovarian granulosa (216) and testicular cells (217) production of steroid hormones. Ovarian IGF-1 has been implicated as a hormone influencing the action of both FSH and LH and almost all known actions of FSH are amplified by IGF-1 (216). Peripherally administered IGF-1 has been shown to stimulate LH secretion in sheep in a dose dependent manner. It seems that IGF-1 is intrinsically stimulatory to LH at low doses, but is inhibitory at higher doses due to hypoglycemia induced by IGF-1 acting at the insulin receptor (218). It seems that estradiol may be important for IGF-1 to inhibit LH secretion. Higher IGF-1 doses (100 and 150 $\mu\text{g/kg}$), did not affect LH secretion in ovariectomized ewes but LH levels decreased by 16-22% in the presence of estradiol (218). *In vitro* studies have shown that IGF-1 is capable of stimulating LH release from cultured ovine pituitary cells independent of GnRH (215). Circulating levels of IGF-1 are elevated at puberty in rodents (213;219), primates (220), and ruminants (221). The onset of puberty is delayed in heifers immunized against GRF (to decrease serum GH and serum IGF-1 concentrations) at three and six months of age (188;222).

Estradiol has been shown to regulate IGF-1 gene expression in several tissues including the uterus (223). In the ovariectomized rat, estradiol increases IGF-1 mRNA and IGFBP-2 mRNA throughout the anterior pituitary while IGF-1 receptor mRNA expression is unchanged (224). However, castration enhances, rather than abolishes the

IGF-1 surge which occurs at puberty (219). This could be due to different actions of estradiol vs. testosterone. Collectively, available evidence points to IGF-1 acting as a hormonal factor that can mediate the effects of inadequate nutrition on gonadotropin secretion.

3.b.) Insulin

Insulin is released from pancreatic β -cells in response to increased glucose levels and is therefore closely associated with nutritional status in an acute sense. Peripheral concentrations of insulin are directly proportional to the level of food intake (225) and body adiposity (226) and thus may serve as a long-term indicator of nutritional state. Insulin enters the CSF from plasma via a receptor-mediated transport system (227). In a variety of species the brain, and in particular the hypothalamus, contains both insulin and insulin receptors (228). Van Houten *et al.* (229) showed that insulin receptors are present in the arcuate nucleus and median eminence in rats. The anatomical location of mRNA for insulin receptor in the hypothalamus provides evidence that insulin acts directly to influence hypothalamic function. Indeed, it has long been known that insulin acts centrally to decrease food intake (230;231). Schechter *et al.* (232) have shown mRNA encoding insulin in the brain of rabbits, indicating a potential role of locally produced insulin.

Insulin also has actions on the reproductive axis. Increases in insulin concentrations in both the peripheral circulation and CSF are associated with increases in gonadotropin secretion (233). *In vitro*, insulin can stimulate the release of GnRH from rat hypothalamic explants (211;234). Low dose infusion of insulin into the third ventricle of rams fed a restricted diet or diabetic male lambs increases LH pulse frequency to

values that are similar to controls (235;236). Withdrawal of insulin from diabetic male lambs previously receiving insulin treatment for 24 hours decreases LH pulse frequency whereas re-supplementation of insulin restores LH pulse frequency. After 96 hrs of insulin withdrawal in these lambs, stimulation of LH pulsatility by insulin replacement was exaggerated (237). In contrast, insulin injection into the lateral cerebral ventricle did not increase LH secretion in growth-restricted, hypogonadotropic lambs (238).

Insulin also affects differentiation of porcine granulosa cells by potentiating basal and gonadotropin-induced progesterone production by enhancing FSH-stimulated LH receptor induction (239). Pharmacological concentrations of insulin enhance basal and LH-stimulated progesterone levels and androstenedione accumulation in cultures of porcine thecal tissue (240). These effects may be mediated by insulin binding to IGF receptors. It should be noted that studies using insulin are often difficult to interpret since insulin influences the concentration of many other hormones and metabolic factors, especially that of glucose.

3.c.) Glucose

Glucose has long been a favored candidate as the metabolic link between nutrition and reproduction (122). Glucose is maintained within a narrow concentration in the blood, as demonstrated by the rapid return of circulating glucose levels to baseline following a postprandial increase. It is the primary metabolic fuel used in ATP production by cells and is the only significant energy-yielding substrate utilized by the brain (241). Thus, glucose levels or availability directly affect activity of the central nervous system (174). In addition, neural activity may also be directly altered by changes in glucose availability since glucose is the precursor for a number of neurotransmitters,

including glutamate, aspartate, GABA, glutamine, and alanine (174). Glucose is sensed centrally with a large body of evidence supporting a role for the brainstem as a glucosensing region. Lesions to the area postrema (AP) and nucleus of the solitary tract (NTS) in the brainstem block hypoglycemia-induced increases in feeding behavior in rats (242). The AP is outside the blood-brain-barrier, so it is well positioned to sense circulating concentrations. Therefore it is possible that glucose availability is detected in the lower brainstem and the information is relayed to rostral structures controlling both GnRH secretion and food intake. However, there is also evidence to suggest that neurons of the VMH and LHA sense glucose (243). The relative role of the brainstem and hypothalamus in glucosensing has yet to be determined and is a matter of some debate.

Glucose utilization and availability affect the functioning of the reproductive axis, and therefore glucose meets the three requirements for a signal linking nutrition and reproduction. Schneider and Wade (173) demonstrated that blockade of glycolysis in combination with fatty acid oxidation resulted in immediate cessation of estrous cycles in obese hamsters. Given the importance of pulsatile LH secretion in control of reproductive activity, it seems plausible to propose that availability of oxidizable metabolic fuels such as glucose or ketones may impact hypothalamic control of LH release. Nutritionally anestrus cows have lower serum concentration of glucose, along with lower insulin and higher nonesterified fatty acids, than cows on a maintenance diet (244). Inhibition of fat and glucose metabolism depresses pulsatile secretion of LH in female lambs (245). In ewes, insulin-induced hypoglycemia inhibits LH secretion and administration of glucose reverses this effect (189;246) and glucose stimulates LH release in growth-restricted wethers (247). Intravenous infusion of 2-Deoxy-D-Glucose

blocked the expression of estrous behavior and ovulation in cows (248) and reduced the magnitude of estradiol-induced surges of LH in anestrus sheep (249). In addition, in rats and lambs, 2DG decreases LH pulse frequency to values typically seen in undernourished animals (250;251). Treatment with 2DG also prevented the occurrence of estrus and the formation of corpora lutea in beef heifers (248). These actions appear to be centrally mediated, since GnRH administration stimulated LH secretion in 2DG-treated animals and controls to a similar extent (189). Collectively these studies lead to the idea that neurons controlling pulsatile release of LH may be sensitive to the availability of oxidizable metabolic fuels (122).

3.d.) Thyroid Hormone

Thyroid hormones are another class of hormones that meet the requirements for a nutritional signal. Nutrition can profoundly alter the synthesis, secretion, peripheral metabolism, and function of thyroid hormones. Weight loss and energy restriction reduces serum T_4 and T_3 in several species (160;252-255). Conversely, during dynamic weight gain serum T_4 and T_3 increases (253;256). There is apparently a change in hypothalamic and/or pituitary responsiveness to feedback inhibition from circulating thyroid hormones during undernutrition since TSH levels remain the same or even drop during starvation (257), and there is a blunted TSH response to TRH (258). Changes in T_3 seen with changes in nutrition seem to involve practically every level of thyroid regulation, beginning in the CNS and ending with the final action of thyroid hormones in the nucleus of cells. The level and composition of energy intake, including whether the organism is in normal energy balance, are important signals directing these hormones

(259). Thyroid hormones are lipophylic and can diffuse through the blood brain barrier, and feedback to control the release of TRH into portal blood.

In some seasonal breeders, the thyroid gland plays an important role in reproductive transitions. For example, thyroidectomy before the breeding season blocks the eventual transition out of the breeding season in European starlings (260;261). This phenomenon has subsequently been observed in other avian species (262) and in sheep (263). Although thyroid hormones do not seem to affect the onset of the breeding season, estrous cycles can be sustained throughout the spring-summer anestrus period in ovary-intact thyroidectomized ewes housed in a fixed photoperiod (263) and the duration of the breeding season can be lengthened by inhibiting T_4 synthesis (264). In the ovariectomized estradiol-treated ewe, pulsatile release of LH (265) and GnRH (266) is maintained at high levels throughout the nonbreeding season if animals have been thyroidectomized during the preceding breeding season. Thus, the steroid-dependent decline in LH pulse frequency that occurs as daylengths increase depends on the presence of thyroid hormones, and thyroidectomy around the time of maximal LH pulse frequency prevents this decline (62). T_4 appears to be essential for the increased response to estradiol negative feedback that causes seasonal anestrus (267). There is no variation, however in plasma TSH in ewes during changes between the breeding and non-breeding season (263). The response to thyroidectomy can be reversed by treatment with T_4 , suggesting that this hormone or one of its metabolites, such as T_3 , mediates the influence of the thyroid on termination of the breeding season (266;268). In addition, constant levels of T_4 are effective, suggesting that it is a change in responsiveness to T_4 or T_3 that allows the onset of anestrus and not changes in T_4 or T_3 *per se*. Results from

experiments in which T_4 was infused into the lateral cerebral ventricles of thyroidectomized ewes and the aforementioned effect of T_4 on GnRH release strongly suggest that they act centrally. However, acute peripheral T_4 administration does not alter GnRH secretion in thyroidectomized ewes (269). Anderson *et al.* (270) tested the response to T_4 of specific areas within the hypothalamus by inserting T_4 containing microimplants into various hypothalamic areas. When T_4 was administered to the premammillary region (PMR), and in some animals to the ventromedial preoptic area (vmPOA), thyroidectomized animals expressed a normal decline in LH at the end of the breeding season. Collectively, these data indicate that the actions of thyroid hormones within specific areas of the hypothalamus are necessary for the transition into seasonal anestrus brought about by increased sensitivity of GnRH release to estradiol negative feedback.

3.e.) Glucocorticoids

There is a strong negative correlation between corticosteroid secretion and nutritional status in humans, rats, and other mammalian species, suggesting that activity of the hypothalamo-pituitary-adrenal (HPA) axis is important for regulating calorie input, disposition, and mobilization (271). The fact that changes in corticosterone levels reflect nutritional status, along with its influence on central food intake and reproduction make glucocorticoids an interesting and compelling nutritional signal controlling reproduction.

The reproductive axis is inhibited at all levels by various components of the HPA axis (272). In particular, glucocorticoids secreted from the adrenal glands inhibit GnRH release, pituitary gonadotrophs, and the gonads. In pursuing the argument that glucocorticoids are paramount in mediating the inhibitory effects of stress on

reproduction, studies have shown that administration of natural or synthetic glucocorticoids can inhibit the secretion of the gonadotropins in sheep (273), pigs (274;275), cattle (276), rhesus monkeys (277), and humans (278). However, it should be noted that other studies have failed to observe an effect of glucocorticoids on LH secretion. Treatment of ovariectomized ewes with dexamethasone for 14 days did not decrease the secretion of LH or inhibit the LH response to GnRH; neither did cortisol nor dexamethasone treatment affect the generation of the LH surge in intact ewes (279). In intact rams, an infusion of cortisol for 9 hours did not affect the LH response to GnRH (280). These differences in LH response to glucocorticoids as compared to those of Juniewicz *et al.* (273) may be due to the short treatment period in Fuquay *et al.* (280) or sex differences in the case of Phillips *et al.* (279).

The influence of cortisol on LH secretion may depend upon steroid milieu. Daley *et al.* (281) reported no change in LH following cortisol administration in castrated rams. However, infusion of castrated rams with a combination of cortisol and estradiol decreased the frequency of LH pulses in these animals more so than estradiol alone. Infusion of estradiol into castrated rams also increased tissue concentrations of GnRH receptor and GnRH receptor mRNA, and cortisol infusion reduced these responses (281;282). These authors speculated that cortisol affects GnRH release by altering its response to estradiol.

The ability of glucocorticoids to affect reproduction also appears to depend upon the duration and route of administration. Application for 7-16 days of acute stressors that activate the HPA axis did not impair reproduction in the pig (283). Consistent with this finding, repeated i.v. injection of cortisol for eight days had no effect on LH secretion in

ovariectomized pigs, even in the presence of estradiol (274). A subsequent study showed that a similar cortisol treatment paradigm did not influence generation of the LH surge, estrous behavior, or ovulation in gilts (275). In contrast, sustained high cortisol concentrations achieved through intramuscular injections of cortisol twice daily for eight days impaired the secretion of LH in ovariectomized pigs in the absence, but not the presence, of estradiol but only on the eighth day of treatment (274). In intact pigs, sustained elevations of cortisol also impaired estrus expression, generation of the LH surge, and ovulation (275). Similarly, monkeys (277) and humans (284) require high doses of cortisol for extended periods of time to impair reproduction. The site of glucocorticoid action on the reproductive axis appears to lie centrally. In intact rams, neither cortisol nor dexamethasone had an effect on either basal or GnRH-stimulated LH (273;280). These results support the concept that corticosteroids do not directly affect secretion of LH by the pituitary, but act at the level of the hypothalamus.

In summary, increased secretion of glucocorticoids is not always associated with decreased secretion of the gonadotropins, particularly in cases of acute stress. Furthermore, there may be species differences in the extent to which glucocorticoids inhibit the secretion of LH. Finally, sex and sex steroid status may influence the effects that glucocorticoids have on GnRH and gonadotropin secretion (285).

3.f.) Leptin

The *ob/ob* mouse has a homozygous mutation in the *obese* gene and is hyperphagic, hyperinsulinemic, hyperglycemic, infertile, exhibits abnormally low energy expenditure, and is morbidly obese (286;287). Based on parabiosis studies, Coleman *et.al.* (288) suggested that *ob/ob* mice lack a blood-borne factor that could

regulate adiposity by modulation of appetite and metabolism. This prediction subsequently proved true as that hormone was cloned and sequenced by Zhang *et.al.* (289) and called leptin, from the greek word leptos, meaning thin. Circulating levels of leptin are derived largely from secretion by adipocytes and this hormone plays an important role in regulating body weight and metabolism (290-292). Plasma leptin levels are proportional to body adiposity (293) and leptin potently reduces food intake, body weight and adipose mass when injected into leptin-deficient or normal mice (290-292). Thus, Zhang *et al.* (289) proposed that leptin acted as an adipostatic hormone whose primary function is to combat obesity. However, leptin levels decrease with fasting (160) and other studies have shown that prevention of the decrease in leptin seen with fasting blunts the activation of the HPA axis and prevents the suppression of the thyroid, reproductive and growth hormone axes (151;160;294-296). Consequently, Frederich *et al.* (297) proposed that leptin acts as a starvation signal. In truth, leptin undoubtedly serves both purposes, though its more predominant actions probably lie in its function as a signal of reduced energy balance.

A large body of evidence suggests leptin can influence reproduction. Mice overexpressing leptin reach puberty earlier, despite a leaner phenotype (298). On the other hand the *ob/ob* mouse, which lacks leptin, is infertile. Infertility from this mutation is believed to be due to reduced circulating gonadal steroids (299), likely due to insufficient hypothalamic-pituitary drive (300). Obesity in another genetic model, the *db/db* mouse, results from the absence of a specific isoform of the leptin receptor important for intracellular signaling (301-303). These mice are totally insensitive to the effects of leptin on food intake. Not surprisingly, these mice, like *ob/ob* mice, are

hyperphagic, show decreased energy expenditure and morbid obesity, and are infertile (287). A leptin receptor mutation similar to the *db/db* mutation in mice, occurs very rarely in humans (304). These individuals are hyperphagic, morbidly obese, and exhibit abnormal pubertal development (304). Thus, without question, the presence of leptin is required for normal reproductive competency.

Whether leptin acts as a signal for puberty onset, versus being merely permissive, is a matter of some debate. Leptin has been implicated in timing the onset of puberty in rodents (305-307), monkeys (308), and humans (309;310). However, work in the rat has shown that leptin did not advance the age of puberty (307), suggesting that leptin is not the primary trigger initiating puberty in this species. Leptin concentrations also do not change at the time of puberty in rats (311) and increase only after increases in LH at puberty in monkeys (112), indicating that leptin does not have the characteristics to be considered a signal for the onset of puberty. Thus, more work is needed to determine the exact nature of leptin's role in the reproduction of well-nourished individuals. It is clear, however, that leptin can restore reproductive function during undernutrition. Following fasting, monkeys (312), prepubertal heifers (313), and rats (314) being treated with leptin had higher mean LH levels than controls and leptin can restore estrous cycles in fasted mice (160).

The signaling form of the leptin receptor (ObRb) has been found at all points along the HPG axis (315). Distribution of ObRb in the brain varies among species, but all species studied thus far express ObRb mRNA in the arcuate nucleus and ventromedial nucleus of the hypothalamus (312), two areas acknowledged to be involved in regulating feeding and reproductive behavior. Leptin has been shown to stimulate GnRH secretion

from hypothalamic explants (316) and reduce fasting-induced decreases in GnRH pulse frequency (314). Yet experiments in the rat (317) and monkey (294) show little co-expression of ObRb and GnRH protein or mRNA, suggesting an indirect mechanism of action for leptin-induced GnRH release. An effect of leptin on reproduction also may be due to its influence on metabolic fuel availability. ObRb is located in gluco-sensitive areas of the hypothalamus and brainstem and leptin has been shown to influence these gluco-sensitive neurons, which could then potentially influence GnRH secretion. Leptin may also work via the GH/IGF-1 axis to mediate GnRH release, as the fasting-induced decrease in GH secretion was prevented by leptin treatment in rats (296;318). Leptin restored both LH and GH secretion in castrate male sheep that had been fasted for 78 hr (319).

Leptin may also have a direct effect on pituitary release of LH. ObRb is expressed in the anterior pituitary and leptin stimulates gonadotropin release from rat anterior pituitary fragments *in vitro* (316). Maciel *et al.* (313) have shown leptin to increase the pituitary's responsiveness to GnRH. In the gonads, leptin has been shown to regulate mRNA expression for side chain cleavage enzyme and 17- α -hydroxylase (315), enzymes involved in steroid synthesis, and to modulate LH-stimulated estradiol production by the ovary (320). Leptin receptors are also present in rodent leydig cells and leptin has been reported to inhibit basal and stimulated testosterone production *in vitro* (321). Leptin levels are negatively correlated with basal and stimulated testosterone levels in humans (322). Leptin may also affect reproduction via the HPA axis. Leptin treatment of wild-type mice prevents fasting-induced increases in corticosterone and suppression of estrous cycles (160).

In summary, leptin is an adipocyte-derived hormone that is critical for both normal body weight regulation and reproductive development. Whether leptin plays an active role in puberty onset or is simply permissive remains to be determined. What seems to be clear is that leptin drops precipitously with undernutrition and leptin replacement restores reproductive function. Thus leptin may be an important “metabolic” hormone linking nutrition with reproduction.

4) Central Integration of Reproductive and Nutritional Cues

4.a.) Hypothalamus

Special subsets of neurons within the hypothalamus are sensitive to circulating nutrients that reach them. There are glucosensitive neurons in the VMH and lateral hypothalamus and some neurons in these areas are responsive to not only glucose but amino acids and fatty acids as well (323). Some hormones, notably leptin and insulin, are actively transported into the brain and receptors for both leptin and insulin are expressed in areas of the hypothalamus important for regulating food intake. Other hormones, such as glucocorticoids, may modulate the responses to these signals. Food deprivation decreases the number of estradiol responsive immunoreactive cells in the VMH and increases numbers of these cells in the Arc and POA (324). The hypothalamus also receives neural input from several areas, notably the nucleus tractus solitarius and area postrema in the brainstem, that relay many neural and hormonal signals from the periphery and/or gastrointestinal tract (325). To influence reproduction, nutritional signals must be transduced into neural inputs that then influence GnRH neuronal activity. Although there are many likely candidates, NPY is a particularly attractive one.

4.a.1. NPY and Food Intake

Neuropeptide Y (NPY), a 36 amino acid member of the pancreatic polypeptide family, was first isolated in 1982 (326;327). In the brain, NPY is expressed at high levels in the arcuate nucleus of the hypothalamus, nucleus accumbens, septum, and the periaqueductal gray matter, as well as in lesser amounts in the hippocampus, amygdala, thalamus and basal ganglia (328). Most of its metabolic effects, however, arise from cells located within the hypothalamus (329).

NPY is one of the most powerful known endogenous stimulators of food intake. NPY neurons found in the Arc that project to the PVN (paraventricular nucleus) are responsible for the stimulatory effects of NPY on feeding behavior (330;331). This member of the pancreatic polypeptide family interacts with at least six distinct G protein-coupled receptors designated Y₁, Y₂, Y₃, Y₄, Y₅, and y₆ (332). The Y₁ receptor has a high affinity for NPY and its mRNA and receptor protein is abundantly expressed in many rat and mouse brain regions, including hypothalamic centers controlling energy homeostasis (333-335). Receptor Y₅ has also been implicated in mediating NPY's effects on food intake, with receptor mRNA discretely localized in the arcuate nucleus and PVN of the rat and mouse brain (334-336). Intracerebroventricular administration of NPY stimulates food intake, presumably via the Y₅ receptor and at least some of the other NPY receptor subtypes in the hypothalamus (337;338).

Acutely, NPY stimulates food intake, even in satiated animals, for hours following central administration (339-342). When administered chronically, NPY induces a state mimicking hormonal and metabolic changes seen in obesity (343). Indeed, repeated (344) and continuous (345) administration of NPY into hypothalamic

sites produces obesity. NPY provides such a strong motivation to eat that NPY-treated animals will carry out operant behaviors such as lever pressing and will endure electric shocks in order to obtain food (346). Both secretion and content of NPY in the PVN increases in rats with a robust drive for feeding following fasting (331). NPY returns to control levels upon initiation of feeding (347) and its release is closely associated with the energy requirements of the rats (331). Studies of ovariectomized sheep living outdoors indicate that changes in the expression of NPY mRNA and NPY peptide levels in the arcuate correlate with seasonal changes in voluntary food intake (64;348).

4.a.2.) NPY and Reproduction

NPY influences GnRH release and potentiates the responsiveness of gonadotrophs to GnRH (349). NPY may also be involved in mediating steroid negative feedback on GnRH release. NPY alters GnRH and LH release (67;146-148) and NPY-containing neurons synapse directly onto GnRH neurons (149). Although GnRH neurons themselves have not been shown to contain ER α or androgen receptors, NPY neurons do coexpress ER α (150). Thus, steroid-induced changes in GnRH and LH release may, at least in part, be due to altered input from the NPY system.

Whether NPY stimulates or inhibits GnRH/LH secretion depends somewhat on the context in which it is examined. Much of the work has been done in females and a large body of evidence suggests that NPY is stimulatory to GnRH release during the follicular phase/preovulatory LH surge. In the rat, NPY mRNA and protein levels increase at the time of the preovulatory LH surge (**350-353**) and reducing NPY input can attenuate or block the LH surge (349;354). Data are more limited in other species, but generally support a stimulatory role for NPY in generation of the GnRH surge for sheep

(355-357), monkeys (358;359), and mice (360). In contrast, the influence of NPY on tonic LH release varies and depends upon the particular species examined. In rats, the role of NPY appears to depend upon the prevailing steroidal environment. NPY inhibits GnRH/LH release in ovariectomized rats but stimulates GnRH/LH release in steroid-treated animals (360-364). In monkeys, perfusion of the median eminence with NPY stimulates GnRH release in males and females regardless of the presence or absence of steroids (148;365). In domestic livestock species, the influence of NPY on tonic GnRH/LH secretion seems to be uniformly inhibitory. NPY inhibits LH secretion in ewes or cows that are ovariectomized or ovariectomized and implanted with estradiol (17;67;115;147;366).

NPY-containing neurons synapse directly onto GnRH neurons (149). One site of action of NPY resides in the mPOA where synaptic links between immunopositive NPY axon terminals and soma containing GnRH have been observed (367). The NPY network also communicates directly or indirectly through other efferents with the GnRH network in the ME-Arcuate as NPY readily stimulates GnRH release from nerve terminals in the ME (361). Thus experimental evidence to date is consistent with the view that the NPY network in the arcuate nucleus/median eminence and the mPOA represents an integral link in the neural circuitry regulating GnRH secretion.

4.a.3.) Seasonal Changes in NPY

Relatively few studies have examined NPY in a seasonal context, but these studies suggest that NPY gene and protein levels change in a season-dependent manner. Hypothalamic NPY-containing cell numbers and NPY mRNA expression is higher during the breeding season in ovariectomized ewes, and estradiol did not influence this

response (348;368). Skinner and Herbison (369) observed that numbers of immunoreactive NPY neurons are increased during the mid-luteal phase of the estrous cycle compared to anestrus. Clarke *et al.* (370) observed that NPY mRNA levels increased during a long-day photoperiod in intact male sheep. While these studies clearly suggest that hypothalamic NPY levels change with daylength, it is not possible to determine the exact stimulus for these changes. It may be that NPY input can be altered directly by changes in photoperiod. Also, differences in NPY levels could potentially be explained by differences in the steroid milieu that prevails during the mid-luteal phase of the estrous cycle (e.g. progesterone) compared to anestrus (e.g. estradiol). Alternatively, since NPY is an important regulator of food intake and metabolism, changes in ambient temperature or other environmental factors could affect expression. Therefore, one aspect of my thesis investigates the relationship between NPY mRNA expression and LH secretion during different photoperiods and steroid environments in male sheep.

4.a.3.) Sex-differences

Sex-related differences exist in response to various energy states and hypothalamic neuropeptides known to be involved in controlling food intake, and potentially linking nutritional changes to reproductive changes. Interestingly, female NPY knock-out mice respond more robustly than male NPY knock-out mice to leptin (371;371) and NPY appears to be responsible for a greater percentage of obesity in females than males when NPY knock-out mice are crossed onto an *ob/ob* background (371). In *ob/ob* mice lacking NPY, one third of the females were fertile compared to only 5% of the males (371). Since *ob/ob* mice are always infertile, this finding suggests that

NPY may play a role in leptin deficient infertility to a greater degree in females than males. NPY-Y2 receptor knock-out mice of both sexes show a decrease in body weight relative to controls, yet food intake is decreased in males and increased in females (372). NPY-Y1 receptor deletion caused a fourfold increase in fat mass as a percentage of body weight in females, but no increase in males.

4.b.) brainstem

In addition to the hypothalamus, the brainstem may play an important role in linking nutrition and reproduction. The area postrema (AP) and nucleus of the solitary tract (NTS), both within the brainstem, transmit metabolic information to hypothalamic areas that influence GnRH release. The AP is ideal for sensing peripheral metabolic information because it lacks a distinct blood-brain-barrier (373). Ritter *et al.* (374) have defined more specific sites within the hindbrain that are glucosensitive. These areas in the dorsomedial region are intimately associated with the fourth ventricle, in close proximity to the AP, and the ventrolateral sites are close to an area on the ventrolateral surface of the medulla that has been shown to have a higher local glucose utilization rate, higher blood flow, and a greater density of perfused capillaries than surrounding medullary tissue (375). These are areas very well suited for sensing peripheral glucose concentrations and neuronal cell bodies in the AP-NTS project to the rostral structures of the brain, including the PVN and lateral hypothalamus, which are implicated in the regulation of feeding and reproduction (376). Therefore it is likely that peripheral metabolic signals are detected here and the information is relayed to rostral structures controlling both GnRH secretion and food intake. Indeed, neural elements of the AP are necessary for glutamate-induced feeding (377).

Caudal brainstem structures have been suggested to mediate the suppressive effect of glucoprivation induced by 2DG on pulsatile LH secretion in the rat. Infusion of very low doses of 2DG into the fourth ventricle will suppress LH secretion in the rat (373). The AP mediates the suppression of pulsatile LH secretion in response to insulin-induced hypoglycemia (378). AP lesions block metabolic inhibitor-induced suppression of estrous behavior as well as the associated decrease in ER immunoreactivity in the VMH (324). These authors suggested that estrogen-binding cells in the VMH, critical for induction of lordosis in hamsters, receive information about metabolic fuel availability via the AP. Similarly, AP lesions block 2DG-induced inhibition of estrous cycles in Syrian hamsters (379). Specifically lesioning catecholaminergic neurons that project from the brainstem to the PVN eliminate the increased length of estrous cycles seen during glucoprivation (380). Thus the brainstem seems to play an essential role during limited glucose availability with regard to inhibiting estrous behavior and expression of estrous cycles, at least in rodent models studied thus far.

Table 1
Effect of neurotransmitters that mediate steroid negative feedback on GnRH/LH secretion

β-endorphin	inhibitory	Barb et al. (381)
Norephrinphrine**	inhibitory	Havern et al. (382), Riggs and Malven (383)
Dopamine*	inhibitory	Lehman et al. (384), Havern et al. (382)
NPY	inhibitory/ stimulatory#	Bauer-Dantoin et al. (146), Woller et al. (148), Barker-Gibb et al. (67), Malven et al. (147)
γ-aminobutyric acid	inhibitory	Ferreira et al. (385;386)
glutamate	stimulatory	Caraty et al. (387)

* May only have effect during anestrus

** NE is necessary for the LH surge to occur

Dependent on species

Chapter 2

NEUROPEPTIDE Y GENE EXPRESSION IN MALE SHEEP: INFLUENCE OF PHOTOPERIOD AND TESTOSTERONE

Abstract

The frequency of pulsatile release of gonadotropin-releasing hormone (GnRH) and LH is high in the breeding season and low in the nonbreeding season. These alterations in the patterns of GnRH and LH release are due to an interaction of daylength and gonadal steroid negative feedback. A vast amount of data indicates that steroid-responsive neural systems play a role in regulating seasonal changes in GnRH release. One candidate system is neuropeptide Y (NPY). To determine the independent and interactive influences of photoperiod and steroid exposure on NPY mRNA levels, we used hypothalamic tissue from four groups (n=4 per group) of castrated male sheep that were simultaneously housed in photochambers and exposed to: 1) a 16L:8D photoperiod (LD), 2) LD and implanted with testosterone (LD+T), 3) a 10L:14D photoperiod (SD), and 4) SD+T. Circulating levels of T averaged 2.8 ± 0.2 ng/ml in implanted animals, but were undetectable in nonimplanted males. Mean LH levels were significantly reduced ($P < 0.01$) in the LD+T group compared to the other groups which did not differ from each other. Silver grain area per NPY neuron in the arcuate nucleus, as assessed by in situ hybridization, was inversely related to mean LH values with grain area per cell being significantly greater ($P < 0.05$) for LD+T males than all other groups, which did not differ from each other. NPY cell numbers were not significantly different ($P > 0.10$) among treatment groups. These results show that NPY expression is increased in male sheep

during a LD photoperiod in a T-dependent manner. Our data are consistent with the idea that NPY is involved in seasonal regulation of GnRH and LH release in the male sheep.

Introduction

Sheep are seasonal breeders that exhibit reproductive activity during the relatively shorter days of fall and early winter and transition into reproductive quiescence during the lengthening days of late winter or early spring (388;388). These seasonal swings in reproductive activity are due to changes in pulsatile patterns of gonadotropin hormone releasing-hormone (GnRH), and thus, luteinizing hormone (LH) secretion. During the breeding season, pulsatile GnRH and LH secretion exhibits a high frequency pattern of release whereas during the nonbreeding season, GnRH and LH secretion is characterized by a low-frequency pattern of release. These alterations in the patterns of GnRH and LH release are due to an interaction of daylength and gonadal steroid negative feedback (389-393). However, the neural mechanisms whereby steroids and photoperiod interact to control GnRH and LH secretion are not completely understood.

Whether steroids directly influence GnRH release by acting directly at GnRH neurons has been a matter of much study. The fact that GnRH neurons apparently contain few, if any, estrogen receptor (ER)- α or androgen receptors (394;395) has led to the idea that gonadal steroids do not act directly to influence GnRH release. However, it has recently been reported that GnRH neurons coexpress mRNA and protein for ER- β (396-399), another ER isoform. This raises the possibility that estradiol may directly influence GnRH neural cell function, although the nature of that effect has not been clearly established in vivo. Clearly, however, steroids influence GnRH release through other steroid-responsive neurons. Neuropeptide Y (NPY), a 36 amino acid peptide

synthesized and prevalent in the arcuate nucleus (400-402), is one candidate. NPY alters GnRH and LH pulsatility (403-406) and NPY-containing neurons synapse directly onto GnRH neurons (407). In addition, NPY neurons coexpress ER α (408). Thus, photoperiod-induced changes in GnRH and LH release may, at least in part, be due to altered input from a steroid-responsive NPY system.

The relatively few studies that have examined NPY in a seasonal context suggest that NPY mRNA and protein levels change in a season-dependent manner. Clarke et al. (409) observed that NPY mRNA levels increased during a long-day photoperiod in intact male sheep. In female sheep, hypothalamic NPY-containing cell numbers and NPY mRNA expression was higher during the breeding season in ovariectomized ewes, and estradiol did not influence this response (410). Skinner and Herbison (411) observed that numbers of immunoreactive NPY neurons in ewes are increased during the mid-luteal phase of the estrous cycle compared to the nonbreeding season. While these studies clearly suggest that hypothalamic NPY levels change with daylength, it is not possible to determine if these changes are due solely to photoperiod or if they may also be influenced by ambient temperature or steroid environment. In order to determine the independent and interactive influences of photoperiod and steroid exposure on NPY mRNA levels, we used hypothalamic tissue from males that were contemporaneously exposed to either long or short daylengths in the presence or absence of testosterone.

Materials and Methods

Tissues used in this study were taken from animals used in a previously published report (412). Twenty black-faced sheep, predominantly of the Suffolk breed, were

housed at the Veterinary Research Farm at the University of Illinois, Urbana (Latitude 40° N) and maintained in light-sealed photochambers that were partially controlled for temperature by air conditioners and heating units. They were fed alfalfa hay and allowed water *ad libitum*. All procedures were carried out in accordance with NIH Guidelines for the Care and Use of Laboratory Animal Care.

Eight males were long-term castrates (>6 months) while 12 were castrated 1 month prior to the beginning of the experiment. Animals were assigned to one of four treatment groups with assignment balanced for time postcastration: 1) short day (8L:16D), no testosterone (SD), 2) short day with testosterone (SD+T), 3) long day (16L:8D), no testosterone (LD), and 4) long day with testosterone (LD+T). The LD groups entered photochambers on September 21 and were subjected to 90 days of 16L:8D followed by 90 days of 8L:16D and then a final 90 days of 16L:8D. The SD groups were left outdoors until December 21 whereafter they were subjected to 90 days of 16L:8D followed by a final 90 days of 8L:16D. LD and LD+T animals were put through successive photoperiod treatments to phase reverse the change in LH secretion so that all animals could be sacrificed at a similar time and ambient temperature, but under contrasting photoregimens.

Animals receiving testosterone were implanted as previously described (413) on December 21. To validate the LH response to photoperiod and determine circulating testosterone levels, blood samples were collected twice weekly via jugular venipuncture during the last 3 weeks of the final photoperiod treatment and assayed for plasma T and LH as previously described (414). At the end of the study, animals were euthanized with an overdose of barbiturate and hypothalami were quickly excised. Tissue was frozen

quickly on dry ice and stored at -70°C until sectioned on a cryostat and used for *in situ* hybridization.

In situ hybridization

Radiolabeled cRNA probes for ovine NPY mRNA were generated from a 311 bp cDNA sequence kindly provided by Dr. Duane Keisler (University of Missouri, Columbia MO). Probes were labeled with ^{35}S -UTP and tissue sections were processed as previously described (23-24). Tissue sections through the arcuate nucleus were hybridized under glass cover slips overnight at 55°C with labeled cRNA (5×10^5 cpm/40 μl hybridization buffer/section). Post-hybridization washes were performed as described by Greco et al. (415). Tissue sections then were dipped in autoradiographic emulsion (NTB3; Kodak, Rochester NY) diluted 1:1 with deionized H_2O . Sections were exposed for approximately 10 days at 4°C , and then developed with Dektol developer and Kodak Fixer. Finally, tissue sections were counterstained with 0.08% Toluidine-O Blue and coverslipped with Depex. Hybridization controls on test tissues included hybridization with labeled ‘sense’ strand cRNA probes. No hybridization signal was detected on these tissues.

Sections of the arcuate nucleus from each animal were anatomically matched according to the arcuate shape and relation to the third ventricle, and a researcher unaware of the treatment groups analyzed two of these sections. Due to the large number of NPY containing cells, only one side of each section was analyzed. Results obtained were an average of these two hemi-sections. Silver grain area above individual neurons was determined at 40X using the public domain program NIH Image (developed at the US National Institutes of Health and available on the Internet at

<http://rsb.info.nih.gov/hin-image/>). Light intensity and threshold were set and maintained for all animals. For each animal, silver grains above individual neurons were digitized on screen and the area occupied by the grains minus the background was determined.

Statistical analysis

NPY silver grain area per neuron and cell number were calculated for each animal and then averaged among animals to derive treatment group means. Values were compared using a two-way analysis of variance with the model including steroid, photoperiod and the steroid x photoperiod interaction. Comparisons between individual treatment groups were performed by the test of Least Significant Difference.

Results

LH and Testosterone

As previously reported (416), LH values were significantly lower ($P < 0.01$) in the LD+T group (all below assay sensitivity of 2.0 ng/ml) compared with all other treatment groups. LH values did not differ ($P > 0.10$) among SD (14.94 ± 1.16 ng/ml), SD+T (15.32 ± 0.66 ng/ml) and LD (16.49 ± 1.16 ng/ml) groups. Circulating testosterone concentrations in testosterone-implanted males were 2.75 ± 0.16 ng/ml. Testosterone levels were below assay sensitivity (1.0 ng/ml) in males without T implants.

NPY mRNA Expression and Cell Numbers

Photomicrographs from individual animals representing each treatment group are shown in Figure 1. Group means for silver grain area per neuron and cell numbers are shown in Figures 2 and 3, respectively. Silver grain area per NPY neuron was inversely related to mean LH values with grain area being significantly greater ($P < 0.05$) for LD+T animals

than all other groups. Grain area did not differ significantly ($P>0.10$) among LD, SD, and SD+T. NPY cell numbers did not differ among groups ($P>0.40$).

Discussion

Our study is the first to examine the independent influences of photoperiod and testosterone on NPY gene expression in the arcuate nucleus of the sheep. The results suggest that testosterone alters NPY gene expression during a long-day, but not short-day, photoperiod. Interestingly, NPY mRNA levels inversely reflected mean LH values in our study, i.e. LH values were reduced only in the group expressing significantly higher NPY mRNA levels. These data suggest that reductions in LH secretion resulting from photoperiod-induced increases in sensitivity to testosterone negative feedback may be mediated, at least in part, by elevated NPY.

The inverse relationship in our study of mean LH values and NPY mRNA expression suggests that NPY inhibits LH secretion. Whether NPY stimulates or inhibits GnRH/LH secretion depends somewhat on the context in which it is examined. Much work has been done in females and a large body of evidence suggests that NPY is stimulatory to GnRH release during the follicular phase/preovulatory LH surge. In the rat, NPY mRNA and protein levels or NPY-Y1 receptor mRNA levels increase at the time of the preovulatory LH surge (417-421) and reducing NPY input can attenuate or block the LH surge (422;423). Data are more limited in other species, but generally support a stimulatory role for NPY in generation of the GnRH surge for sheep (423-425), monkeys (426;427) and mice (428). In contrast, the effect of NPY on tonic LH release varies and depends upon experimental conditions. In rats, the role of NPY appears to depend upon

the prevailing steroidal environment since NPY inhibits GnRH/LH release in ovariectomized rats but stimulates GnRH/LH release in steroid-treated animals (429-433). In monkeys, perfusion of the median eminence with NPY stimulates GnRH release regardless of steroid milieu (434-436). Interestingly, NPY delivered to the third ventricle inhibits GnRH release (437) suggesting that the influence of NPY on GnRH release may be site-specific in this species. Consistent with our data, the influence of NPY on tonic GnRH/LH secretion seems to be uniformly inhibitory in domestic livestock species. NPY inhibits LH secretion in ewes or cows that are ovariectomized or ovariectomized and implanted with estradiol (438-443). This is consistent with the elevation in NPY gene expression normally observed during food restriction in both male (444) and female sheep (445), periods that are characterized by low levels of tonic LH secretion, and agrees with our observation that NPY mRNA levels were inversely related to mean LH levels.

To our knowledge, ours is the first report to examine the effect of testosterone within short and long days on NPY mRNA expression in domestic livestock species. Clarke et al. (446) observed that arcuate NPY mRNA levels increased during long days in intact male sheep. We also observed an increase in NPY gene expression during long days, but only in response to testosterone, suggesting that photoperiod by itself is not a sufficient stimulus. Our data are consistent with previous work showing that testosterone stimulates NPY. In humans, NPY levels decrease with antiandrogen treatment (447) and increase in women with polycystic ovarian syndrome who exhibit increased circulating testosterone levels (448). In rats, castration reduces and testosterone stimulates NPY mRNA and protein levels (449-454). However, our data are at odds with that observed

for monkeys wherein castration or treatment with testosterone does not affect NPY mRNA levels (455;456), suggesting that species differences exist in the effects of testosterone on NPY input.

There appears to be a significant sexual dichotomy in the regulation of NPY by photoperiod and steroids. As mentioned above, testosterone stimulates NPY gene and protein expression in male rats and we found that NPY mRNA levels increased only in LD+T animals. In contrast, work in the ewe (457) shows that seasonal changes in NPY mRNA or protein expression are steroid-independent and actually increase during short days, e.g. the breeding season. Furthermore, NPY levels in female rats were observed to be only half that of male rats (458) and several studies have reported an inhibitory effect of estradiol on NPY mRNA or peptide levels in female rats (459-462). Thus, it may be that NPY mRNA and protein levels are suppressed in the estradiol-dominated female while they are stimulated in the androgen-dominated male. Sexually dimorphic NPY levels may, at least in part, be due to the metabolism of testosterone to dihydrotestosterone (DHT) via the enzyme 5 α -reductase in males. We previously showed that conversion of testosterone to DHT is an important step in inhibition of LH secretion by testosterone (463). While the influence of DHT on NPY has not been directly assessed, like testosterone, DHT prevents cold-induced loss of body weight (464), increases body mass (465), and increases food intake (466;467) raising the possibility that DHT may mediate, at least in part, metabolic responses involving NPY that are influenced by testosterone.

Although our data are consistent with the idea that daylength-induced alterations in NPY input may play a role in regulating seasonal fluctuations in tonic LH secretion

and fertility, they are also consistent with the notion that changes in NPY input underlie seasonal alterations in voluntary food intake. Castration reduces and testosterone stimulates body weight gain, nitrogen retention, and/or food intake in a number of species (468-478). Intact male sheep exhibit increased numbers of cells expressing NPY and increased NPY mRNA during long days that are associated with increased voluntary food intake (479). Furthermore, NPY is a well known, potent stimulator of food intake in several species including sheep (480). Thus, an increase in NPY mRNA expression in response to testosterone during long days may play a role in increasing voluntary food intake in male sheep.

Most likely, however, is that NPY is involved in regulating both reproduction and food intake. Nutrition and reproduction are heavily intertwined and NPY may be one neuropeptide system linking one with the other. In male sheep, photoperiod alters androgenic input to GnRH neurons so that the ability of testosterone to suppress reproduction is maximal during long days. Based on our data, we would suggest that NPY may be part of this inhibitory pathway, while fulfilling a dual role by stimulating voluntary food intake during this time of year when food availability is greatest.

Figure 1

Figure 2

Figure 3

Figure Legends

Figure 1. Darkfield photomicrographs (10X) of the arcuate nucleus of the hypothalamus taken from representative animals for SD, SD+T, LD, and LD+T. Sections were exposed to a cRNA antisense NPY probe corresponding to nucleotides ----- of the ovine NPY sequence. 3V – third ventricle.

Figure 2. NPY mRNA expression as assessed by silver grain area per neuron (μM^2) for SD, SD+T, LD, and LD+T animals. NPY silver grain area was elevated ($P < 0.05$) only in LD+T animals, and SD, SD+T, and LD groups did not differ significantly ($P > 0.10$). One side of each of two arcuate sections were assessed per animal and values represent the mean $\pm$ SEM of 4 animals per group.

Figure 3. Numbers of NPY cells per animal for SD, SD+T, LD, and LD+T animals. Cell numbers did not differ significantly ($P > 0.10$) among treatment groups. One side of each of two arcuate sections were assessed per animal and values represent the mean $\pm$ SEM of 4 animals per group.

Chapter 3

INFLUENCE OF INSULIN-LIKE GROWTH FACTOR-1 (IGF-1) ON THYROXINE (T₄) AND TRIIODOTHYRONINE (T₃) IN FOOD-DEPRIVED MALE AND FEMALE SHEEP

Abstract

Three experiments were performed to test the hypothesis that IGF-1 alters circulating levels of total T₄ and total T₃. For the first two experiments, we used ewes 9 months of age, each of which was ovariectomized and implanted sc with a 1 cm estradiol implant. In Experiment 1, ewes were food-deprived for 6 days with 3 blood samples collected every 2h on Day 0 prior to food removal and on days 2, 4, and 6 of food withdrawal. Beginning on Day 4, ewes received subcutaneous injections of saline (controls; n=5), or IGF-1 at either 50 ug/kg/d (n=4) or 10 ug/kg/d (n=5) twice daily (0800 and 2000 h). For all studies, we used long R3-IGF-1, an analog with reduced affinity for IGF-1 binding proteins. Six days of food deprivation lowered total T₄ and total T₃ levels in controls. IGF-1 elevated both total T₄ and total T₃ in a dose-dependent fashion by Day 6 of food withdrawal. In Expt 2, females were food deprived for 6 days and then given intravenous injections of IGF-1 at either 25 ug/kg (n=5), 12.5 ug/kg (n=5), or 6.25 ug/kg (n=5). Blood samples were collected at 12-min intervals for the subsequent hour and another sample collected at 2h postinjection. IGF-1 increased total T₄ values significantly in all groups at 60 and 120 min postinjection, but increased total T₃ in all groups by 36 minutes postinjection. In Experiment 3, castrated males that were 9 months old and implanted

with a 1 cm estradiol implant were food deprived for 4 days. During this period, they received either saline injections (control; n=5) or IGF-1 at 25 (n=5), 50 (n=5) or 100 (n=5) ug/kg/d given subcutaneously. Food deprivation decreased total T₄ and total T₃ levels. IGF-1 was able to maintain total T₄ and total T₃ levels for 2 days, after which total T₄ and total T₃ concentrations fell to levels that were less than those on Day 0, but still greater than those observed in controls. In summary, IGF-1 clearly increased circulating levels of both T₄ and T₃, but was more potent in females than in males.

Introduction

For most species, food supply is not constant. Instead they are often exposed to periods of energy abundance and scarcity. To maintain homeostasis and a constant supply of nutrients to the brain, these swings in food availability are met by a variety of compensatory changes in endocrine and metabolic parameters (481). One such change occurs in circulating levels of T₄ and T₃, which are important for determining basal metabolic rate (482;483). Weight loss and energy restriction reduces serum T₄ and T₃ in several species (160;252-255). Conversely, during dynamic weight gain serum T₄ and T₃ increases (253;256). This response likely represents a compensatory mechanism to slow or speed, respectively, metabolism in order to help maintain energy balance. The mechanisms that regulate T₄ and T₃ levels during periods of altered energy balance are not completely known. One possibility is that they may be influenced by peripheral signals that reflect metabolic status, such as insulin-like growth factor-1 (IGF-1).

IGF-1 is synthesized in many tissues including brain, hypothalamus, pituitary, and liver (185-187). Secretion of IGF-1 from the liver in response to pituitary-derived growth hormone (GH) accounts for the secretion of approximately 75% of circulating IGF-1

(484). IGF-1 acts in endocrine, paracrine, and autocrine fashions as an integral component of multiple systems controlling growth, differentiation, and metabolism (200;201). IGF-1 is markedly affected by the nutritional status of an animal, decreasing during periods of negative energy balance (204;485) and increasing during periods of positive energy balance (486). Thus, circulating levels of IGF-1 reflect energy balance.

Reports of the effects of GH, or IGF-1, on the hypothalamo-pituitary-thyroid (HPT) axis are relatively few, and mostly gathered from human patients with somatotrophic aberrations. GH-deficient adults (487), and children with either GH deficiency (488) or hypopituitarism (489) demonstrate decreased serum T_4 and increased serum T_3 levels. (490) have shown that administration of IGF-1 quickly decreases serum TSH and causes a progressive decline in serum free T_4 while T_3 levels are maintained in GH-resistant patients. Thus, while it is likely that IGF-1 influences thyroid hormone secretion, the site(s) at which IGF-1 may act is unknown. While the aforementioned data suggests an action at the pituitary, affects at the hypothalamus or thyroid gland itself cannot be ruled out. Furthermore, the above studies used individuals fed a normal diet and so the relationship between IGF-1, T_4 , and T_3 during food deprivation has not been examined. One purpose of these studies is to test the hypothesis that IGF-1 stimulates T_4 and T_3 secretion in food-deprived animals.

Males and females respond differently to changes in energy state. It has been hypothesized that due to gender-specific evolutionary selection pressures, female mammals have evolved a greater capacity to conserve energy than males (152). Accordingly food restriction has been reported to have a greater and more permanent effect on physical growth in males than in females and in mature humans and animals,

males are more susceptible to the adverse effects of starvation than females (491). Thus, a second purpose of this work is to determine if the thyroid hormone response to IGF-1 is sex-dependent.

Materials and Methods

General

All sheep used in these studies were of Suffolk or Dorset breeding and were approximately 9 months of age. They were housed indoors in light (14L:10D) and temperature controlled rooms and fed *ad libitum* a diet consisting of alfalfa pellets supplemented with cracked corn. Animals had unlimited access to water at all times. All procedures were approved by the West Virginia University Animal Care and Use Committee and followed NIH guidelines for use of animals in research.

Experiment 1

The purpose of this experiment was to determine if chronic administration of IGF-1 stimulates T_4 and T_3 secretion in food-deprived females. Fourteen ewes were ovariectomized two weeks prior to the beginning of the study. At the time of ovariectomy, each ewe received a length of silastic tubing (Sil-Med, Tri-anim, Sylmar, CA; inner diameter 3.35mm, outer diameter 4.65mm) packed with 1 cm of crystalline β -estradiol (Sigma-Aldridge Inc., St Louis MO) and closed at both ends with silicone sealant. Two weeks prior to the study, animals were group-housed with each pen holding five animals. Ewes were provided a normal meal on the morning of Day 0 and allowed time to eat after which they received no food for the remainder of the 6-day period of food withdrawal. Animals were allocated to three treatment groups with assignment balanced for body weight on Day 0: 1) food-deprived control (n=5), 2) food-deprived and receiving 10ug/kg/d IGF-1 (IGF-10; n=5), and 3) food-deprived and receiving 50ug/kg/d

IGF-1 (IGF-50; n=4). Animals receiving IGF-1 were administered long R3-IGF-1 (GroPep Ltd, Adelaide, Australia), an IGF-1 analog with reduced affinity for IGF-1 binding proteins, subcutaneously twice daily at 0800 h and 2000 h beginning on Day 4 of food removal. Weights were recorded at 0800 h and three blood samples were collected via jugular venipuncture every 2 hours over a 4-hour period beginning at 0900 h on days 0, 2, 4, and 6. Samples were allowed to sit overnight, were centrifuged, and the serum was removed and frozen at -20°C. Samples were analyzed for total T₄, total T₃, insulin, and glucose.

Experiment 2

The purpose of this study was to determine if IGF-1 acutely stimulates total T₄ and total T₃ secretion. Similar to Experiment 1, ovariectomized ewes were food deprived for 6 days. On Day 6, IGF-1 was administered as a single intravenous bolus at a dose of 6.5 ug/kg (n=5), 12.5 ug/kg (n=5), or 25 ug/kg (n=5). Blood samples were collected every 12 minutes from just prior to IGF-1 injection (0 minutes) to 60 minutes following IGF-1 injection, and a single sample was collected 120 minutes post-injection. Blood samples were processed as above and total T₄ and total T₃ were assessed for each sample.

Experiment 3

The purpose of this experiment was to determine the effect of chronic IGF-1 administration on T₄ and T₃ secretion in food-deprived males. Two weeks prior to the study, 28 wethers were each implanted with a 1 cm estradiol implant and were group-housed with each pen holding 6 animals. Wethers were provided a normal meal on the morning of Day 0 and allowed time to eat after which they received no food for the remainder of the 4-day period of food withdrawal. Wethers were divided into four

treatment groups initially balanced for body weight on Day 0: 1) food-deprived control group (n=6), 2) food-deprived and receiving 25 ug/kg/d IGF-1 (IGF-25; n=6), 3) food-deprived and receiving 50 ug/kg/d IGF-1 (IGF-50; n=6), or 4) food-deprived and receiving 100ug/kg/d IGF-1 (IGF-100; n=5). Animals receiving IGF-1 were administered long R3-IGF-1 (GroPep Ltd, Adelaide, Australia) subcutaneously twice daily at 0800 h and 2000 h beginning on day 1. Weights were recorded at 0800 h and blood samples were collected via jugular venipuncture every 2 hours over a 4-hour period beginning at 0900 h on days 0, 2, and 4. Samples were allowed to sit overnight, were centrifuged, and the serum was removed and frozen at -20°C. Samples were analyzed for total T₄, total T₃, insulin, and glucose.

Hormone Assays

Total T₄ was assessed using a commercially available kit from Diagnostic Products Corporation (Los Angeles, CA). For males, all samples within an experiment were run in duplicate within one assay. The intraassay coefficient of variation was 10.6% with sensitivity being 0.1 ng/ml. For females, three assays were performed with sensitivity being 0.1 ng/ml and interassay and intrassay coefficients of variation being 14.3% and 7.0%, respectively. Total T₃ was assessed using a commercially available kit from Diagnostic Products Corporation. For males, all samples within an experiment were run in duplicate within one assay. The intraassay coefficient of variation was 4.2% with sensitivity being 2 ng/dl. For females, 2 assays were performed with sensitivity being 2 ng/dl and interassay and intrassay coefficients of variation being 7.2% and 5.5%, respectively. Insulin was assessed using a commercially available kit from Linco Research Inc. (St. Charles, MO). All samples within an experiment were run in duplicate

within one assay. The intraassay coefficient of variation was 6.6% with sensitivity being 0.1 ng/ml. Serum glucose levels were measured spectrophotometrically in singlet using the Trinder reagent kit and performed according to manufacturer's instructions (Sigma Diagnostics Inc., St. Louis MO).

Statistical Analysis

Values for individual samples were averaged to provide a mean for each animal within each blood collection period (or day). Data for body weight, total T₄, total T₃, insulin, and glucose were analyzed by two-way ANOVA for repeated measures to determine the effects of time, dose of IGF-1, and their interaction. Those groups showing a significant time x dose interaction were further analyzed by one way analysis of variance followed by a test of least significance difference to determine influence of IGF-1 dose among groups within each day, and by one way repeated measures followed by a Student-Newman-Keuls test to determine the effect of time within each group over the 4 or 6 day treatment period for males or females, respectively. In addition, for Experiment 2, Dunnet's method was used to compare post-injection time period means to the control period (0 minutes).

Results

Experiment 1

As expected, food deprivation caused a significant ($P < 0.05$) decrease in body weight (Figure 1). The percent change in body weight did not differ among groups ($P > 0.10$). Collectively, ewes lost $14.4 \pm 1.56\%$ of their initial body weights over the 6-day period of food withdrawal.

Changes in total circulating T_4 and T_3 in ewes are illustrated in Figure 2A and 2B, respectively. As expected, circulating levels of both T_4 and T_3 were decreased ($P<0.05$) in controls by food deprivation. In contrast, treatment with IGF-1 elevated total circulating T_4 levels by day 6 of food removal in a dose dependent manner. Total T_4 levels for IGF-50 ewes were elevated ($P<0.05$) above their Day 0 values and were greater ($P<0.05$) than Day 6 values for controls. Values for IGF-10 ewes were returned to their Day 0 values by IGF-1 treatment and were intermediate of controls and IGF-50 ewes on Day 6. Likewise, IGF-1 increased circulating T_3 in a dose-dependent manner. Total T_3 levels in all groups differed significantly ($P<0.05$) from each other on day 6 and total T_3 was returned to Day 0 values in IGF-10 ewes and exceeded Day 0 values in IGF-50 ewes.

As expected, insulin concentrations (Figure 3A) decreased ($P<0.01$) in food-deprived females. Insulin levels were lower ($P<0.05$) in IGF-1 treated groups compared to controls on days 4 and 6, though there was no significant difference ($P<0.10$) between IGF-1 treated groups. Glucose levels (figure 3B) dropped slightly, but significantly ($P<0.01$) over time in food-deprived controls. Effects of IGF-1 on circulating glucose levels were dependent upon dose. Glucose dropped ($P<0.01$) over time in the IGF-10 group, but values were not different than controls ($P>0.05$). Glucose levels were lower ($P<0.05$) on days 4 and 6 in the IGF-50 group than either controls or the IGF-10 group.

Experiment 2

Total T_4 values in ewes receiving intravenous injections of IGF-1 are shown in Figure 4A. ANOVA revealed a significant effect of period ($P<0.001$), but no significant effect of dose ($P>0.60$) or dose x period interaction ($P>0.60$). Total T_4 levels were increased ($P<0.05$) over 0 minute control values at 60 and 120 minutes post-injection.

Total T₃ values in ewes receiving intravenous injections of IGF-1 are shown in Figure 4B. ANOVA revealed a significant effect of period ($P<0.001$), but no significant effect of dose ($P>0.30$) or dose x period interaction ($P>0.20$). Total T₃ concentrations were increased ($P<0.05$) over 0 minute control values at 36, 48, 60 and 120 minutes post-injection.

Experiment 3

As expected, food removal for 4 days resulted in a significant ($P<0.001$) loss of body weight (Figure 5). The percent change in body weight did not differ ($P>0.80$) among treatment groups with males losing $12.49 \pm 0.90\%$ of their initial body weight over the 4 day food withdrawal period.

Total T₄ values (Figure 6A) in controls progressively decreased ($P<0.05$) on each day of food removal. Compared to day 0 values, Total T₄ values on day 2 for IGF-25 and IGF-50 males were not significantly different ($P>0.10$), but were increased ($P<0.05$) slightly in IGF-100 males. Total T₄ levels decreased ($P<0.05$) in all IGF-1 treated groups on day 4 compared to day 2. However, total T₄ was significantly ($P<0.01$) higher for all IGF-1 treated groups than controls on day 2 and remained so on day 4. Total T₃ levels (Figure 6B) progressively declined ($P<0.05$) in both control males and IGF-25 animals. Total T₃ in the IGF-50 males did not differ from Day 0 levels until Day 4 of food removal ($P<0.05$). Although total T₃ levels decreased in IGF-100 males over time, this reduction was not statistically significant ($P>0.10$). Total T₃ levels did not differ ($P>0.20$) between treatment groups on Day 2. On Day 4, total T₃ values were higher ($P<0.05$) in IGF-50 and IGF-100 males than in control males. Total T₃ levels in IGF-25 wethers were

intermediate of those in controls and IGF-50, i.e. levels in IGF-25 males were not significantly different ($P>0.05$) from either control or IGF-50 males.

Insulin levels (Figure 7A) dropped below assay sensitivity by day 2 of food removal and remained undetectable in all groups. Circulating glucose levels (Figure 5b) decreased ($P<0.05$) in control, IGF-25, and IGF-50 males on day 2 and 4 compared to day 0. In the IGF-100 males, circulating glucose concentration on days 0 and 2 were similar ($P>0.05$), but glucose levels dropped significantly on day 4 ($P<0.05$). Glucose levels among groups were not significantly different ($P>0.05$) on Day 2. Glucose levels were similar ($P>0.05$) between IGF-50 and IGF-100 males on Day 4 and both were significantly ($P<0.05$) lower than either controls or IGF-25 males, which did not differ from each other ($P>0.05$).

Discussion

In order to determine the effect of IGF-1 on circulating thyroid hormone concentrations, we administered exogenous IGF-1 to food-deprived sheep in three separate experiments, two using females and one using males. The results from these studies support our hypothesis that IGF-1 increases thyroid hormone levels during food deprivation. Furthermore, results from these experiments show that food deprivation has a greater impact on males than females and that the thyroid hormone response to IGF-1 during food deprivation is much greater in females. These data are consistent with the idea that females are much more able to cope with energy perturbations than males and further suggest that changes in IGF-1 may provide a signal linking nutritional status with basal metabolic rate through an alteration in circulating thyroid hormone levels.

Animals on the current study lost 12% (males) and 14% (females) of their initial body weight over a four or six day period of food deprivation, respectively. Although it is difficult to directly compare the males and females in our studies since the experiments were performed in differing years, the rate of weight loss appeared to be very similar for males and females and consistent with previous work (319). In keeping with past reports in several species, food deprivation caused a significant decrease in total T_4 and T_3 (160;252-255). Circulating concentrations of total T_4 appeared to be higher on day 0 in males than in females and decreased more dramatically in males than females with food deprivation. In contrast, circulating levels of total T_4 increased in IGF-1 treated females, reaching Day 0 levels in ewes treated with 10 ug/kg/d and exceeding Day 0 levels in ewes treated with 50 ug/kg/d. Females also demonstrated a dramatic and dose-related increase in total circulating T_3 levels following IGF-1 treatment. In contrast, IGF-1 treated males were able to maintain total T_4 levels until Day 2, after which circulating T_4 dropped. Nonetheless, values in IGF-1 treated males remained above control values suggesting that IGF-1 has the ability, albeit limited, to influence thyroid hormone levels in food deprived males. Likewise, IGF-1 appeared to slow, in a dose-dependent fashion, the decrease in T_3 levels observed with fasting in males. Based on these data, we would conclude that IGF-1 is a more potent stimulus of thyroid hormone levels in females than in males.

The site of action at which IGF-1 stimulates thyroid hormone secretion as well as the mechanism whereby this occurs is at present unknown. Very few studies have addressed how IGF-1 or its secretagogue, GH, influence the HPT axis. Theoretically, IGF-1 could influence thyroid hormone levels by acting at all levels of the HPT axis

since IGF-1 receptor protein or mRNA has been localized to the hypothalamus and pituitary (189), and the thyroid gland (492). To our knowledge, there is no data available as to whether IGF-1 influences TRH release. A limited number of reports suggest that GH or IGF-1 can inhibit pituitary TSH release (487;493-495). Klinger et al. (490) showed that either a bolus i.v. or subcutaneous injection of IGF-1 reduced TSH levels and proposed that this was due to increased somatostatin release. However, another study by Trainer (496) observed decreased TSH levels after IGF-1 treatment without significant changes in GH release and the author suggested that the suppression in TSH was due to elevated T_4 and T_3 . Our data are consistent with this explanation. A direct action of IGF-1 at the pituitary is suggested by studies showing altered sensitivity to TRH stimulation following GH or IGF-1 treatment (488;497;498). We did not measure TSH in our study. TSH is difficult to detect in serum collected from thyroid-intact sheep (Dr. Fred Karsch, personal communication), a fact confirmed by our attempt to measure TSH with a commercially available assay. Further study will be needed to address the issue of where IGF-1 acts to increase thyroid hormone levels.

In both females and males, circulating levels of total T_3 appeared to follow closely serum levels of total T_4 . This is not too surprising since at least 75% of all circulating T_3 is derived from T_4 by peripheral 5'-deiodination rather than from thyroid gland secretion (257;499;500). The finding for females that total T_3 levels increased prior to total T_4 levels in our second experiment suggests that IGF-1 stimulates deiodinase activity. This is consistent with previous work indicating that GH or IGF-1 treatment stimulates the conversion of T_4 to T_3 . Salamon et al. (501) observed that GH-deficient adults treated with GH and who were on fixed doses of T_4 replacement therapy developed

thyrotoxicosis associated with a rise in plasma T_3 values. Grunfeld et al. (502) reported that normal subjects treated with GH exhibited a decrease in TSH and T_4 , but increased T_3 levels. Other studies have also reported increased T_3 but decreased T_4 (487;488), or increased T_3 without a change in T_4 (489;495) following hGH or IGF-1 administration. Alternatively, increased T_3 levels may have simply been due to increased precursor T_4 levels without a change in deiodinase activity. Despite the fact that T_3 levels increased within 1 hour of IGF-1 administration, total T_4 levels remained constant, suggesting that T_4 secretion likely was also being stimulated at this time. Indeed, total T_4 levels increased significantly once total T_3 levels reached their maximum. Thus, the IGF-1-induced increase in total T_3 levels observed for females in our study may result from an increase in precursor T_4 levels and/or an increase in deiodinase activity.

Most of the T_3 and T_4 that is transported in blood is bound to thyroid hormone binding proteins (503;504). Since only the unbound, or free, hormones are able to enter and affect cells, bound T_3 and T_4 is inactive until being released as free hormone into plasma (505). Since we measured total T_3 and T_4 in circulation, we did not assess whether free T_3 or T_4 changed with IGF-1 treatment. However, we would speculate that our measurements of total T_3 and T_4 are of biological relevance for at least two reasons. One is that in previous work examining the effect of GH or IGF-1 on thyroid hormone release, the change in total hormone reflected that of free hormone (487;495;506). Two, previous studies have shown no effect of GH administration on serum thyroglobulin levels (487) or capacity (489).

As expected, insulin decreased following food deprivation. However, the change in insulin appeared to be influenced by both gender and IGF-1 treatment. In control

females, insulin levels decreased yet were clearly detectable on day 4 of food withdrawal and did not change through Day 6. However, concentrations of insulin decreased to assay sensitivity by Day 2 of food removal in control males. This is consistent with the idea that males respond more robustly than females to food removal. On days 4 and 6 of food removal, both IGF-10 and IGF-50 females exhibited lower circulating insulin concentrations than control females. For IGF-50 females, this drop in insulin may be related to an accompanying drop in blood glucose levels, perhaps due to the insulin-like actions of IGF-1 (507;508). However, glucose levels in controls and IGF-10 females were similar, suggesting that IGF-1 increased insulin sensitivity at this lower dose. This is consistent with a previous study wherein IGF-1 decreased circulating insulin levels although there was no change in glucose concentrations (494). In males, since insulin levels dropped so quickly it was not possible to determine if IGF-1 had any effect on insulin levels.

In summary, though body weight loss following food removal was relatively similar between males and females, changes in circulating insulin, total T_4 , and total T_3 were sex-dependent. Insulin, T_4 , and T_3 levels decreased more quickly and to a greater degree in food-deprived males than in food-deprived females. These results suggest that males react more strongly to food deprivation and that females may be better able to deal, in an energetic sense, with this type of perturbation in energy supply. Both sexes demonstrated an increase in T_3 and T_4 over controls following IGF-1 treatment. Total T_4 and T_3 levels in females were increased to or beyond Day 0 control values after three days of IGF-1 treatment. In contrast, during IGF-1 treatment, total T_4 and T_3 levels were maintained in males for 2 days, after which they declined significantly. This response

was observed even though males received similar or an even higher doses of IGF-1 as the females. Based on these data, we would conclude that IGF-1 is a more potent stimulus of thyroid hormone levels in females than in males. Further study is needed to determine the mechanisms which underlie this sex-dependent response to IGF-1.

Figure 1

Figure 2

Figure 3

Figure 4

A

B

Figure 5

Figure 6

Figure 7

Figure 1

A) Percent change from Day 0 body weight on Day 2 and Day 4 of food removal in controls or wethers receiving IGF-1 subcutaneously twice daily at 25ug/kg/d, 50ug/kg/d, or 100ug/kg/d beginning on Day 1. Weight loss increased over time ($P<0.001$), but did not differ among the four groups ($P>0.80$). B) Percent change from Day 0 body weight on Day 2, Day 4, and Day 6 of food removal in controls or ewes receiving IGF-1 at either 10ug/kg/d or 50ug/kg/d subcutaneously twice daily beginning on Day 3. Weight loss did not differ ($P>0.10$) for the three groups.

Figure 2

Circulating levels of Total T_3 and Total T_4 during 6 days of food removal in controls or ewes receiving IGF-1 subcutaneously twice daily at 10ug/kg/d or 50ug/kg/d beginning on Day 3. A) Total T_4 in control animals differed from IGF-1 treated groups on Day 2 ($P<0.006$) and Day 4 ($P<0.007$). Total T_4 increased from Day 0 levels on Day 2 in animals treated with 100ug/kg/d IGF-1 ($P<0.05$) and decreased in all groups on Day 4 when compared to Day 2. B) Total T_3 levels in all groups did not differ significantly on Days 0, 2, or 4 ($P>0.05$), but were significantly different from each other ($P<0.05$) on Day 6.

Figure 3

Insulin and glucose levels during 6 days of food removal in controls or ewes receiving IGF-1 at 10ug/kg/d or 50ug/kg/d subcutaneously twice daily beginning on Day 3. A) Insulin levels in control ewes decreased from initial levels on Day 2 and remained consistent at that level for the remainder of the treatment period. Insulin levels were not significantly different between treated groups ($P>0.05$) but were lower ($P<0.05$) in IGF-1 treated groups compared to controls on Days 4 and 6 of fasting. B) Glucose did not differ between 10ug/kg/d and control groups, and remained the same throughout the treatment. Day 4 and 6 glucose values were lower than Day 0 and 2 values ($P<0.05$), and were lower than either of the other groups on Days 4 and 6 ($P<0.05$).

Figure 4

Circulating levels of Total T_3 and Total T_4 during 4 days of food removal in controls or wethers receiving IGF-1 at 25ug/kg/d, 50ug/kg/d, or 100ug/kg/d subcutaneously twice daily beginning on Day 1. A) Total T_4 decreased in control animals on each day of the experiment. All treated groups maintained T_4 at the same level between Days 0 and 2, but decreased significantly on Day 4 ($P<0.05$). B) Total T_3 levels decreased from Day 0 levels by Day 4 in control and 25ug/kg/d animals only ($P<0.05$). On Day 4 Total T_3 levels in control animals differed from 50ug/kg/d and 100ug/kg/d ($P<0.05$). 25ug/kg/d were also different from 100ug/kg/d treated groups ($P<0.05$). All groups decreased from Day 0 values by Day 4.

Figure 5

Insulin and glucose levels during 4 days of food removal in controls or wethers receiving IGF-1 at 25ug/kg/d, 50ug/kg/d, or 100ug/kg/d subcutaneously twice daily beginning on Day 1. A) Insulin levels decreased to undetectable levels in all groups by Day 2 ($P<0.001$). B) Glucose levels maintained in control animals throughout the experimental period and decreased by for day0 values on Day 4 in all IGF-1 treated groups ($P<0.05$).

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