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Regulation of GnRH Pulsatility in Ewes

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Abstract

Early work in ewes provided a wealth of information on the physiological regulation of pulsatile GnRH secretion by internal and external inputs. Identification of the neural systems involved, however, was limited by the lack of information on neural mechanisms underlying generation of GnRH pulses. Over the last decade considerable evidence supported the hypothesis that a group of neurons in the arcuate nucleus that contain kisspeptin, neurokinin B, and dynorphin (KNDy neurons) are responsible for synchronizing secretion of GnRH during each pulse in ewes. In this review, we describe our current understanding of the neural systems mediating the actions of ovarian steroids and three external inputs on GnRH pulsatility in light of the hypothesis that KNDy neurons play a key role in GnRH pulse generation. In breeding season adults, estradiol (E_2) and progesterone decrease GnRH pulse amplitude and frequency, respectively, by actions on KNDy neurons, with E_2 decreasing kisspeptin and progesterone increasing dynorphin release onto GnRH neurons. In pre-pubertal lambs, E_2 inhibits GnRH pulse frequency by decreasing kisspeptin and increasing dynorphin release, actions that wane as the lamb matures to allow increased pulsatile GnRH secretion at puberty. Less is known about mediators of undernutrition and stress, although some evidence implicates kisspeptin and dynorphin, respectively, in the inhibition of GnRH pulse frequency by these factors. During the anoestrus, inhibitory photoperiod acting via melatonin activates A15 dopaminergic neurons that innervate KNDy neurons; E_2 increases dopamine release from these neurons to inhibit KNDy neurons and suppress the frequency of kisspeptin and GnRH release.

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Declaration of interest

The authors have no conflict of interest that could be perceived as prejudicing the impartiality of the research present in this review.

Introduction

It is now generally recognized that the episodic nature of gonadotropin-releasing hormone (GnRH) secretion is a key characteristic of hypothalamic control of reproductive function. Episodic release of GnRH was first inferred from the pulsatile pattern of luteinizing hormone (LH) concentrations in ovariectomized (OVX) rhesus monkeys (Dierschke *et al.* 1970), a pattern that has since been observed in many species under a large variety of endocrine conditions (Karsch 1987). Subsequent measurements of GnRH concentrations in the hypophysial portal blood of ewes have demonstrated that this pattern of secretion consists of relative brief (about 5 min long) bouts of GnRH secretion followed by prolong (ranging from 30 min to several hours depending on the endocrine status of the animal) periods when no secretion occurs (Clarke & Cummins 1982, Moenter *et al.* 1992b). The one exception to this episodic pattern occurs during the preovulatory GnRH/LH surge that occurs late in the follicular phase of the ovarian cycle; although acute fluctuations in GnRH levels (Clarke 1993, Evans *et al.* 1995b) have led some to suggest that pulsatile GnRH secretion occurs during the surge (Clarke 1995), it is clear that no prolonged hiatus in GnRH release, similar to that seen during classical episodic secretion, occurs during the GnRH surge (Moenter *et al.* 1992a, Clarke 1993). Moreover, episodic and surge secretions are controlled by different neural networks and feedback actions of ovarian steroids. It is generally accepted in rodents that different neural networks are involved; for example, arcuate kisspeptin neurons participate in the control of GnRH pulses, while the more rostral kisspeptin population mediates the estrogen-induced GnRH surge (Dungan *et al.* 2006). In ewes, arcuate kisspeptin neurons participate in the control of both episodic and surge secretion (Smith *et al.* 2009, Merkley *et al.* 2012), while the more rostral kisspeptin population is only involved in the surge (Merkley *et al.* 2012, Ezzat *et al.* 2015). In these (and other) species, GnRH pulses are controlled by the negative feedback actions of both estradiol (E_2) and progesterone, while the GnRH surge is triggered by the positive feedback action of high E_2 concentrations at the end of the follicular phase of the ovarian cycle. This review will focus on episodic GnRH secretion in ewes that occurs at other times of the oestrous cycle.

The critical importance of pulsatile GnRH secretion was demonstrated in a classic paper (published 40 years ago) which reported that LH and FSH concentrations in OVX monkeys could be maintained with episodic, but not continuous, administration of exogenous GnRH (Belchetz *et al.* 1978). This observation, which has since been confirmed in several other species (Chakraborty *et al.* 1974, Marshall & Kelch 1986, Blum *et al.* 2000), led to the extensive use of long acting GnRH agonists in clinical reproductive medicine. Contemporaneous studies also indicated that changes in GnRH pulse patterns play a major role in timing the events of the ovarian cycle (Goodman & Karsch 1981) and are key determinants of fertility during the pubertal transition (Wildt *et al.* 1980, Ojeda *et al.* 1983, Foster *et al.* 1984) and annually in seasonal breeders (Karsch *et al.* 1980). Thus the importance of episodic GnRH secretion to the neuroendocrine control of reproduction has been established for several decades. Despite this importance, the neural mechanisms responsible for the synchronization of GnRH secretory activity necessary for episodic release remained a “black box” commonly referred to as the “GnRH pulse generator” until

the last ten years. Starting in 2007, several lines of evidence have supported the hypothesis that a group of neurons in the arcuate nucleus (ARC) of the hypothalamus containing kisspeptin, the tachykinin, neurokinin B (NKB), and the endogenous opioid peptide (EOP), dynorphin, (termed KNDy neurons) are responsible for the generation of GnRH pulses (Navarro *et al.* 2009, Lehman *et al.* 2010, Rance *et al.* 2010, Okamura *et al.* 2013).

Studies in ewes have made major contributions to our understanding of the physiological importance of changes in GnRH pulse patterns, the control of these patterns by the inhibitory actions of estradiol and progesterone, and the role of KNDy neurons in pulse generation for two major reasons. First, because sheep have a large blood volume and adapt readily to handling, they are ideally suited for the frequent blood sampling necessary to characterize pulsatile LH secretion under a variety of conditions. More importantly, sheep are the only species in which GnRH concentrations in hypophysial portal blood samples can be monitored for long periods of time in unanesthetized, unstressed animals (Clarke & Cummins 1982, Caraty & Locatelli 1988). Such studies have provided direct evidence of the effects of internal (e.g., ovarian steroids (Clarke *et al.* 1987, Evans *et al.* 1995a)) and external (e.g., stressors) (Battaglia *et al.* 1997, Wagenmaker *et al.* 2009) factors on GnRH secretion, and the best characterization of secretory dynamics of GnRH secretion during each pulse (Moenter *et al.* 1992b). Moreover, KNDy neurons were first identified in ewes (Goodman *et al.* 2007) and this animal model has provided critical experimental evidence supporting the role of KNDy neurons in GnRH pulse generation. Therefore, this review will focus largely on studies in ewes on the control of pulsatile GnRH secretion. We will first briefly describe the current model for GnRH pulse generation, including current unresolved questions. We will then consider the physiological regulation of GnRH pulsatility in sheep under a variety of conditions; in each section, we will summarize earlier studies on this regulation and then discuss our current understanding of the underlying neural mechanisms in the context of the KNDy model for GnRH pulse generation.

The role of KNDy neurons in GnRH pulse generation in sheep

The KNDy model of pulse generation was developed based on several key anatomical characteristics of KNDy neurons in the ovine ARC: 1) as noted above, they contain two stimulatory (kisspeptin and NKB) peptides, both of which are critical for fertility in humans (de Roux *et al.* 2003, Seminara *et al.* 2003, Topaloglu *et al.* 2009), and one inhibitory (dynorphin) peptide (Goodman *et al.* 2004); 2) they form an extensive inter-connected network (Foradori *et al.* 2006); 3) they contain receptors for NKB (NK3R) (Amstalden *et al.* 2010), but not for kisspeptin (*Kiss1r*) (Smith *et al.* 2011); and 4) based on triple-labeled immunohistochemical analysis using unique markers for KNDy neurons, they project to 45% of the GnRH cell bodies in the preoptic area (POA) and to 60% of mediobasal hypothalamus (MBH) GnRH neurons (Merkley *et al.* 2015). It should be noted that projections from neurons in the ARC to POA GnRH cells were not observed in two ewes using anterograde tract tracers (Pompolo *et al.* 2001), but this could reflect the limited number of cells bodies labeled with such tracers (Goodman *et al.* 2018). Moreover retrograde tract tracing studies indicate that dynorphin-ir neurons in the ARC project to the region of the POA containing GnRH cell bodies (Dufourny *et al.* 2005) In its simplest form the KNDy model for GnRH pulse generation (Navarro *et al.* 2009, Lehman *et al.* 2010,

Goodman & Inskeep 2015) proposes that kisspeptin is the output of the KNDy network that drives GnRH secretion during a pulse, while NKB and dynorphin act within this network to initiate and terminate each GnRH pulse, respectively (Figure 1). At the start of a pulse, initial release of NKB triggers a positive feedback loop that rapidly increases KNDy neural activity, kisspeptin release, and thus GnRH secretion. Within minutes dynorphin release begins to hold KNDy neural activity in check and, after about five minutes, terminates kisspeptin release and the GnRH pulse.

Because several recent reviews are available describing the development of, and experimental support for, this model in detail (Okamura *et al.* 2013, Goodman *et al.* 2014, Goodman *et al.* 2015), we will briefly summarize evidence for it in sheep. First, as mentioned above and discussed in more detail later, the cellular location of receptors for KNDy peptides (Figure 2) is consistent with the proposed actions of each peptide. Second, the ability of iv naloxone (a non-specific antagonist to EOP receptors) to increase the amplitude and duration of GnRH release during pulses indicates a role for an EOP in termination of each GnRH pulse (Goodman *et al.* 1995). Third, ARC microimplants of NKB and a NK3R antagonist increased and decreased LH pulse frequency, respectively, while ARC microimplants of a κ-EOP receptor (KOR) antagonist increased LH pulse frequency in OVX ewes (Goodman *et al.* 2013). Finally, assessment of KOR internalization as an index of dynorphin release indicated that dynorphin release begins shortly after the start of a pulse and is still evident toward the end of GnRH secretion (Weems *et al.* 2016a, Weems *et al.* 2017b). This working model has also received substantial experimental support from studies examining bursts in multi-unit activity (MUA) associated with LH pulses in goats (Wakabayashi *et al.* 2010, Okamura *et al.* 2013) and evidence in a number of species that the stimulatory actions of NKB on LH secretion are mediated by kisspeptin released from KNDy neurons (Navarro *et al.* 2011a, Navarro *et al.* 2011b, Ramaswamy *et al.* 2011, Grachev *et al.* 2012)

Although there is now strong evidence that KNDy neural activity is necessary for episodic GnRH secretion in ewes, several unresolved issues remain. First, it is unclear whether episodic kisspeptin release drives GnRH pulses or if kisspeptin is simply permissive for episodic GnRH secretion in ewes. There is direct evidence in monkeys for episodic kisspeptin release in the MBH (Keen *et al.* 2008), and in mice for episodic activation of KNDy neurons just before the onset of each LH pulse (Clarkson *et al.* 2017). In ewes, Fos expression (a commonly used marker for neural activity) increases in KNDy neurons 30 min after an endogenous LH pulse (Ezzat *et al.* 2015), but there is no direct evidence coupling this to episodic kisspeptin release. In the absence of such data, several studies have examined the response of LH (and one study of GnRH) to continuous kisspeptin infusions to indirectly address this issue. Initial work demonstrated that prolonged infusions of kisspeptin were unable to maintain elevated LH concentrations so that there was no difference between treated and control ewes after two days of infusion (Caraty *et al.* 2007). This, and similar data in monkeys (Ramaswamy *et al.* 2007), was interpreted to mean that episodic kisspeptin release is needed for episodic GnRH secretion. More recently, two studies examined this question in a more acute time frame (3–6 hrs), but reached opposite conclusions. In one study, kisspeptin infusions were able to maintain LH pulses during inhibition of episodic LH secretion with an NK3R antagonist in OVX ewes (Clarke *et al.*

2018). In the other, kisspeptin infusions produced prolonged continuous elevations in both GnRH and LH secretion in anoestrous ewes, whereas a bolus kisspeptin injection produced a GnRH and LH pulse (Caraty *et al.* 2013). The most likely explanation for the different LH responses in these two studies is the method used to eliminate endogenous GnRH pulses. The latter study used anestrous ewes, in which KNDy neural activity is strongly inhibited (see below), while the former used an NK3R antagonist to inhibit this neural network. It is possible that the NK3R antagonist did not completely abolish KNDy neural activity in these OVX ewes, particularly since small increments in LH, that did not reach the limit of detection of the pulse analysis used, are evident is some individual ewes in this report. If so, the small residual activity of these neurons on GnRH release might have been amplified by the kisspeptin infusion. Overall, the preponderance of the kisspeptin infusion and Fos data (Ezzat *et al.* 2015) support an episodic release of kisspeptin in ewes, but more direct evidence is needed to resolve this issue.

Another important question involves the specific neurons on which KNDy peptides act to control GnRH secretion during a pulse. That NKB acts on KNDy neurons seems clear because most ovine KNDy neurons, but no GnRH neurons, contain NK3R (Figure 2) (Amstalden *et al.* 2010). Similarly, kisspeptin specifically acts on GnRH neurons because *Kiss1r* is found in GnRH, but not KNDy, neurons in ewes (Smith *et al.* 2011). However, *Kiss1r* is found in non-KNDy neurons in the ovine ARC (Smith *et al.* 2011) and local administration of a KISS1R antagonist produces a modest reduction of LH pulse frequency in OVX ewes (Goodman *et al.* 2013). Thus it is possible that kisspeptin actions within the ARC via non-KNDy neurons contribute to episodic GnRH secretion. In contrast to NK3R and *Kiss1r*, KOR is found in both KNDy and GnRH neurons (Weems *et al.* 2016b) so dynorphin could act on either (or both) cell type to terminate GnRH secretion. As noted above, based on internalization of KOR, dynorphin appears to act on KNDy neurons throughout most of a pulse. Interestingly, KOR internalization was also observed in MBH GnRH neurons but only at the end of a pulse (Weems *et al.* 2017b). No internalization of KOR was observed in POA GnRH neurons, which is consistent with earlier evidence that MBH GnRH neurons drive episodic LH secretion (Boukhliq *et al.* 1999). Thus dynorphin actions on KNDy neurons likely hold GnRH secretion in check during a pulse, and its actions on both KNDy and GnRH neurons contribute to pulse termination.

Control of Pubertal GnRH/LH secretion through KNDy neurons

In the ewe, puberty onset is heralded by an increase in pulsatile GnRH/LH secretion, evident as an increase in LH pulse frequency during the 12 days before the first ovulation (Huffman *et al.* 1987). A large body of evidence indicates that the prepubertal hiatus in GnRH/LH secretion is due to the strong inhibitory actions of E_2 (Foster & Hileman 2015). Thus removal of steroid-negative feedback by OVX in the pre-pubertal ewe lamb results in an elevation of GnRH/LH secretion, which can readily be reduced with subcutaneous administration of E_2 (OVX+E lambs). This E_2-induced suppression reflects an inhibition of GnRH/LH pulse frequency that persists until the time when puberty onset would normally occur (Foster & Hileman 2015). Many other species (e.g., cattle, guinea pigs, and ferrets) share a similar mechanism for control of puberty onset; however, in primates the gonadostat hypothesis does not apply to the entirety of the pre-pubertal period, but does arise during the

peri-pubertal period between menarche and the first ovulation (Goodman 2015). Since circulating levels of E_2 remain constant in this steroid-clamp model, it is the sensitivity to the inhibitory actions of E_2 in the hypothalamus that lessens as the animal matures and allows for the elevation in episodic GnRH/LH secretion necessary for first ovulation (commonly referred to as the gonadostat hypothesis (Foster & Hileman 2015)). While GnRH neurons are the final common output from the central nervous system controlling gonadotropin release from the anterior pituitary, these neurons in the ewe do not express oestrogen receptor-α (Herbison *et al.* 1993, Lehman & Karsch 1993), the receptor thought to mediate the negative feedback of E_2. Therefore, the neural pathways regulating puberty onset in the ewe must lie upstream of GnRH neurons.

In humans (de Roux *et al.* 2003, Seminara *et al.* 2003, Topaloglu *et al.* 2012) and mice (Seminara *et al.* 2003, d'Anglemont de Tassigny *et al.* 2007), disruption of kisspeptin signaling results in blockade of pubertal development. Although similar mutations have not been found in sheep, there is strong support in ewes for the idea that kisspeptin acts as a gatekeeper to puberty onset. Hourly administration of kisspeptin to pre-pubertal ewe lambs acutely stimulated episodic LH secretion and prolonged hourly treatment induced an LH surge and ovulation (Redmond *et al.* 2011b). Thus any central signaling components between kisspeptin and GnRH neurons that are needed for normal GnRH secretion appear to be intact and waiting for the stimulatory kisspeptin drive prior to puberty. ARC kisspeptin expression in the pre-pubertal ewe is substantially reduced by even low levels of circulating E_2 in gonad-intact (Nestor *et al.* 2012) or OVX+E (Lopez *et al.* 2016) lambs compared to OVX lambs. Furthermore, the elevation in LH secretion that occurs around the time of puberty in the ewe is closely mirrored by an increase in both ARC kisspeptin mRNA expression in OVX+ E_2 ewes (Redmond *et al.* 2011a) and kisspeptin protein expression in gonad-intact ewes (Nestor *et al.* 2012, Polkowska *et al.* 2017). There is also an increase in the percentage of POA GnRH neurons that receive kisspeptin input that parallels the change in ARC kisspeptin cell numbers during this period (Nestor *et al.* 2012). Taken together, these data indicate that the increase in pulsatile secretion of GnRH/LH secretion responsible for puberty onset in the ewe is dependent on an increase in signaling by ARC kisspeptin neurons.

Similar to kisspeptin signaling, NKB is critical for pubertal development in humans (Topaloglu *et al.* 2009, Gianetti *et al.* 2010) and there is some evidence that it may stimulate GnRH/LH secretion during ovine puberty onset. In ewe lambs the NK3R agonist, senktide, stimulated LH secretion prior to puberty onset (Nestor *et al.* 2012). NKB expression is also inhibited by the ovary, presumably via E_2 secretion, prior to puberty since the number of NKB-immunoreactive (ir) neurons in the ARC increased following OVX in pre-pubertal lambs. (Nestor *et al.* 2012). However, a similar OVX-induced increase in NKB expression was also seen in post-pubertal lambs, suggesting that this action of E_2 may not change during puberty. Although the number of NKB-ir soma did not change following puberty onset in ovary-intact lambs, we did observe an increase in NKB-ir fiber density in the ARC and suggest that this translates to an increase in NKB activity in post-pubertal lambs (Nestor *et al.* 2012). Thus, although this correlational evidence indicates that increases in NKB contribute to puberty onset in ewes, further work is needed to adequately test this hypothesis. For example, it would be important to determine if there are increases in NKB

input to key reproductive neurons (GnRH, kisspeptin, etc) during puberty and if disruption of NKB signaling delays puberty onset in the ewe lamb.

While kisspeptin and NKB are stimulatory, the third peptide in KNDy neurons, dynorphin, has an inhibitory effect on GnRH/LH secretion. Dynorphin signaling in the adult ewe is known to mediate progesterone-negative feedback on GnRH/LH secretion (Goodman *et al.* 2004, Foradori *et al.* 2005), and there is now growing evidence that this EOP may be part of the "brake" restraining GnRH/LH release prior to puberty in sheep. GnRH neurons in the pre-pubertal ewe lamb express KOR, but dynorphin-ir is not detectable in lambs, although it is readily seen in tissue from adult luteal phase ewes (Lopez *et al.* 2016). Despite this lack of evidence for detectable dynorphin protein expression in lambs, infusion of nor-BNI, a KOR antagonist, into the lateral ventricle of OVX+E lambs at a pre-pubertal age increased LH pulse frequency, but failed to do so in the same lambs at a post-pubertal age (Figure 3). However, it is unclear where dynorphin acts to hold LH in check in pre-pubertal lambs because nor-BNI had no effect on LH secretion when administered into the POA alone or the ARC alone (Figure 3). The two most likely explanations for the differences in effects of nor-BNI in pre-pubertal lambs are: 1) dynorphin action in either the POA or ARC is sufficient to suppress GnRH/LH secretion, or 2) dynorphin acts outside of the POA and ARC to inhibit GnRH/LH secretion.

While the negative feedback action of E_2 is the primary internal signal holding GnRH pulse frequency in check prior to puberty, energy intake is a critical external signal necessary for normal pubertal development. A clear example of this is seen in pre-pubertal ewes where undernutrition to the point of growth restriction delays puberty onset (Fitzgerald *et al.* 1982, Foster & Olster 1985, Prasad *et al.* 1993). This effect of food restriction reflects an inhibition of LH pulse frequency and does not require E_2 because it is evident in OVX lambs (Foster & Olster 1985), although food restriction does also increase the response of the MBH to the inhibitory actions of E_2 (McManus *et al.* 2005). Subsequent work clearly demonstrated that the inhibitory actions of food restriction in OVX lambs reflects a central suppression of GnRH release from the hypothalamus (Ebling *et al.* 1990, Prasad *et al.* 1993, I'Anson *et al.* 2000), but the neural mechanisms by which energy balance regulates GnRH/LH secretion in ewe lambs still remain largely unknown. Early work focused on the possible role of the EOP, β-endorphin, and neuropeptide Y (NPY) because both inhibited LH secretion in sheep (McShane *et al.* 1992, Prasad *et al.* 1993), probably via mu-opioid receptors (Weems *et al.* 2018) and Y2 receptors (Barker-Gibb *et al.* 1995), respectively. Chronic food restriction of ewe lambs increased mRNA encoding NPY in the ARC (McShane *et al.* 1993), but did not alter NPY release in the median eminence (Prasad *et al.* 1993). In contrast, food restriction decreased both the mRNA encoding β-endorphin and the release of this EOP into the median eminence (McShane *et al.* 1993, Prasad *et al.* 1993). However, the relevance of these changes to the inhibition of GnRH release by food restriction is unclear because, as noted above, β-endorphin inhibits GnRH secretion (Prasad *et al.* 1993) and naloxone has no effect on LH secretion in food restricted lambs (Ebling *et al.* 1990).

Considerable effort has been spent trying to identify the peripheral signal(s) controlled by changes in nutritional intake that contribute to the inhibition of GnRH secretion (Foster & Hileman 2015), with a large body of work focused on the adipose-produced hormone leptin.

Most of this work has been done in adults ewes, but one study in gonadectomized lambs reported that leptin concentrations fall with fasting and that exogenous leptin can restore the fasting-induced decrease in LH pulse frequency seen in these animals (Nagatani *et al.* 2000). However, this work was done in castrated ram lambs and the sexes may differ in their response to food restriction (Foster & Hileman 2015). In light of the role of kisspeptin in puberty onset in many species, the recent focus of studies on food restriction in the ewe lamb has started to shift to the role of this peptide. One study demonstrated that two months of food restriction reduced hypothalamic kisspeptin mRNA expression (Wang *et al.* 2016) and another found that a short term (3 day) fast in peri-pubertal ewe lambs reduced kisspeptin protein expression in the POA and ARC (Polkowska *et al.* 2015). Thus studies of the neural mechanisms by which undernutrition inhibits GnRH secretion in lambs are in their early stages and further work is needed to determine the effects of nutrition on NKB and dynorphin expression and the functional roles of all three KNDy peptides in the changes in GnRH pulsatility during times of altered nutritional balance.

Negative feedback control of GnRH pulsatility during the ovine estrous cycle

When considering the negative feedback control of pulsatile GnRH secretion in ewes it is important to distinguish between the breeding season, when regular 16–17 day oestrous cycles occur, and the non-breeding season, when ewes are anovulatory, because there are marked seasonal differences in the actions of E_2 on GnRH pulses (Legan *et al.* 1977, Goodman & Inskeep 2015); these will be discussed in the next section. There are two major negative feedback actions of progesterone and E_2 on GnRH pulses during the ovine oestrous cycle: progesterone inhibits GnRH pulse frequency and E_2 inhibits GnRH pulse amplitude (Evans *et al.* 1995a, Goodman *et al.* 2002). These actions, which account for the changes in GnRH and LH pulse patterns observed in the luteal and follicular phases, were first inferred from LH pulse patterns in jugular blood, and have since been confirmed by measurements of GnRH in hypophysial portal blood (Clarke *et al.* 1987). Estradiol also increases GnRH pulse frequency, alters the shape of GnRH pulses, and increases GnRH secretion between pulses (Evans *et al.* 1995a, Goodman *et al.* 2002). However, since these changes occur with E_2 levels that induce a GnRH/LH surge, but not basal E_2 concentrations, they most likely reflect the positive feedback actions of this steroid. Moreover, there is very little information on the underlying neural mechanisms, (Goodman *et al.* 2002) and no data on relevant changes in KNDy neurons that could account for these three actions of E_2 on pulsatile GnRH secretion. Therefore, we will focus on the negative feedback actions of progesterone and E_2 on GnRH pulse frequency and amplitude.

There is now considerable evidence that progesterone negative feedback is mediated, at least in part, by dynorphin release from KNDy neurons onto MBH GnRH neurons. Early data that led to this hypothesis has been reviewed (Goodman *et al.* 2002) and includes evidence that: 1) knife cuts between the POA and MBH did not affect progesterone negative feedback (Whisnant & Goodman 1994) indicting that the MBH contains all the neural elements sufficient for this action of progesterone; 2) relatively non-selective antagonists to EOP receptors increased LH pulse frequency in luteal phase ewes and in OVX ewes treated with

progesterone, but not in untreated OVX animals (Whisnant & Goodman 1988, Yang *et al.* 1988), observations that have since been confirmed with GnRH measurements (Horton *et al.* 1987, Goodman *et al.* 1995); and 3) the increase in LH pulse frequency induced by an EOP receptor antagonist in luteal phase ewes was associated with increased Fos expression in MBH, but not POA, GnRH neurons (Boukhliq *et al.* 1999). The observation that microimplants in the MBH containing an antagonist selective for KOR, but not those containing an antagonist against the μ- or δ-EOP receptors, increased LH pulse frequency in luteal phase ewes (Goodman *et al.* 2004) focused interest on dynorphin, the endogenous ligand for KOR. Subsequent work demonstrated that progesterone increased dynorphin concentrations in CSF collected from the third ventricle (Foradori *et al.* 2005), that almost all dynorphin neurons in the ARC and periventricular region of the POA contained the classical progesterone receptor (PR) (Foradori *et al.* 2002) that mediates the negative feedback actions of this steroid in ewes (Skinner *et al.* 1998), and that ARC PR-containing dynorphin neurons project to the region of the POA in which GnRH cell bodies are found (Dufourny *et al.* 2005). Because local administration of the PR antagonist, RU486, in the ARC increased LH pulse frequency in progesterone-treated OVX ewes, but RU486-containing microimplants had no effect when placed in the POA, it was concluded that ARC dynorphin (i.e., KNDy) neurons are the site of progesterone negative feedback (Goodman *et al.* 2011). It should be noted, however, that local administration of progesterone to the ARC did not inhibit LH secretion in OVX, or OVX+E, ewes (Goodman *et al.* 2011). It is possible that progesterone from these microimplants did not affect a sufficient number of KNDy neurons to produce a measurable decrease in LH secretion, or that the negative feedback actions of this steroid may involve other neurons/neurotransmitters in addition to KNDy neurons/dynorphin. One possible candidate is a population of neurons in the rostral ARC that contain both orphanin-FQ and PR since an antagonist to orphanin-FQ increased LH pulse frequency in luteal phase ewes (Nestor *et al.* 2013). Another possibility is the quarter of β-endorphin neurons in the ARC that contain PR and project to the POA (Dufourny *et al.* 2005). If these are involved, then they most likely act on POA GnRH neurons because a μ-receptor antagonist (Goodman *et al.* 2004) or β-endorphin antisera (Weesner & Malven 1990) acted in the POA, but not MBH, to increase LH secretion in luteal phase ewes. However, this may be a redundant system since connections between the POA and MBH are not needed for progesterone negative feedback (Whisnant & Goodman 1994). However, this input may be important for the estrogen-induced LH surge in ewes (Walsh & Clarke 1998).

Theoretically, progesterone suppression of kisspeptin or NKB release from KNDy neurons could also contribute to the inhibition of GnRH pulse frequency. The ability of KISS1R (Smith *et al.* 2011) and NK3R (Fraser *et al.* 2015, Li *et al.* 2015) antagonists to inhibit LH pulse frequency in OVX ewes is consistent with this possibility. However, there is no evidence that progesterone affects NKB expression, and little evidence that it affects kisspeptin expression, in ewes. There is one report that progesterone decreased *Kiss1* (determined using in situ hybridization [ISH]) in ewes that were OVX for 5–7 weeks (Smith *et al.* 2007), but this steroid does not inhibit LH secretion in chronically OVX ewes (Karsch *et al.* 1977). In contrast, no effect of progesterone was seen on *Kiss1 mRNA* (measured using qPCR) when treatment began at the time of OVX; as expected, progesterone inhibited LH pulse frequency in these animals, but there was no correlation between LH pulse

frequency and *Kiss1* levels (Weems *et al.* 2018). Thus, the negative feedback action of progesterone appears to be mediated by dynorphin release from KNDy neurons, although non-KNDy neurons may also contribute to this inhibition.

The role of different KNDy peptides in mediating the negative feedback actions of E_2 appears to be more complex than the relatively simple story for progesterone negative feedback. Early studies, based on LH measurements, supported a role for EOP because the same EOP receptor antagonists that increased LH pulse frequency in the presence of progesterone increased LH pulse amplitude in follicular phase and oestrogen-treated OVX ewes, but usually did not in untreated OVX ewes (Figure 4) (Whisnant & Goodman 1988, Yang *et al.* 1988, Goodman *et al.* 2002). However, direct measurements of GnRH did not support this hypothesis; the EOP receptor antagonist, naloxone, produced approximately a 3-fold increase in GnRH pulse amplitude in both OVX and OVX+E ewes (Figure 4) (Goodman *et al.* 1995). Thus the neurotransmitter mediating oestrogen negative feedback in ewes remained unclear until the discovery of the reproductive actions of kisspeptin.

The hypothesis that E_2 inhibited GnRH pulse amplitude in ewes by inhibiting kisspeptin release from KNDy neurons was initially proposed based on studies in rodents (Dungan *et al.* 2006). However, strong evidence for this hypothesis was soon developed in ewes. First, virtually all KNDy neurons contain ERα (Franceschini *et al.* 2006). Second, OVX increased both the number of kisspeptin-ir neurons in the ARC (Smith *et al.* 2008) and the percentage of these neurons that contained Fos (Merkley *et al.* 2012). Third, oestrogen treatment decreased Fos expression in ARC kisspeptin neurons (Smith *et al.* 2009), and consistently inhibited *Kiss1r* expression (using ISH or qPCR) and kisspeptin-ir cell numbers in the ARC, but increased kisspeptin expression in the POA (Smith *et al.* 2007, Smith *et al.* 2008, Weems *et al.* 2017a). Finally, there was a positive correlation between *Kiss1r* expression and LH pulse amplitude (but not frequency) in OVX ewes receiving either no treatment or steroid implants that produced luteal phase concentrations of progesterone and/or E_2 (Weems *et al.* 2018). While all these correlational studies support a role for ARC kisspeptin in E_2 negative feedback, there are only a few functional tests of this hypothesis in ewes. Varied results have been observed with icv administration of KISS1R antagonists to OVX ewes, with some researchers observing a decrease in LH pulse amplitude in response to p-234 (Roseweir *et al.* 2009), but others reporting inhibition of LH pulse frequency by the closely related p-271 (Smith *et al.* 2011). One possible explanation for this apparent discrepancy is that the former study used a lower dose (40 μg/hr of p-234) than the latter (300 μg/hr of p-271). Analysis of episodic LH patterns from one of our previous experiments (Goodman *et al.* 2012) confirmed that 40 μg/hr of p-271 inhibits LH pulse amplitude (from 9.2 ± 1.4 to 4.8 ± 1.3 ng/mL; $P<0.05$, n=4) without affecting interpulse interval (before: 64.5 ± 12.3 min; during: 64.0 ± 7.5 min); a more dramatic effect on pulse amplitude was observed in two ewes that received 60 μg/hr of this antagonist (a decrease from 13.9 ± 1.6 to 2.9 ± 0.1 ng/mL), with again no change in interpulse interval. Thus there is limited functional data and very strong correlational evidence supporting the hypothesis that a decrease in kisspeptin from KNDy neurons mediates E_2 inhibition of GnRH pulse amplitude in ewes, but additional functional studies are needed.

Whether changes in NKB and/or dynorphin contribute to the inhibition of GnRH pulse amplitude remains unclear. Changes in ARC NKB expression in response to changes in ovarian steroids parallel those of kisspeptin (Figure 4): NKB increases with OVX and decreases with oestrogen treatments (Weems *et al.* 2017a). Moreover, the ability of antagonists to NK3R to inhibit LH pulse amplitude in OVX ewes is consistent with this hypothesis (Fraser *et al.* 2015, Clarke *et al.* 2018). Decreases in LH pulse frequency were also seen in these studies, but a strong inhibition of GnRH pulse amplitude might lower LH pulse frequency if LH pulse amplitudes fall below the detection limit of the method used for pulse identification. Thus an E_2-induced inhibition of NKB release could contribute to inhibition of GnRH pulse amplitude. There have been fewer studies on the possible role of dynorphin from KNDy neurons in oestrogen negative feedback because of earlier data indicating that E_2 did not affect the naloxone-induced increase in GnRH pulse amplitude. However, the recent report that E_2 produced an increase in the number of dynorphin-ir cells in the ARC of OVX breeding season ewes (Figure 4) has provided new support for this hypothesis (Weems *et al.* 2017a). In this regard, the earlier study examining GnRH pulse amplitude did observe that the naloxone-induced increase in pulse amplitude persisted after the end of naloxone infusion in OVX+E, but not OVX, ewes (Figure 4) (Goodman *et al.* 1995), suggesting a possible effect of E_2 on the dynamics of dynorphin release. Thus further examination of the possible role of dynorphin in E_2 negative feedback in ewes is warranted.

Finally, it is interesting to note that the KNDy model for GnRH pulse generation provides a simple mechanism by which changes in NKB and/or dynorphin could contribute to the negative feedback action of E_2. There is evidence that dynorphin holds GnRH secretion in check during a pulse (Goodman *et al.* 1995, Weems *et al.* 2016a); if this action is mediated by a decrease in kisspeptin, one would predict that a slight increase in dynorphin would produce a decrease in kisspeptin release leading to lower GnRH pulse amplitude. Similarly there is compelling evidence, including the cellular location of NK3R in ewes (Figure 2), that NKB stimulates GnRH secretion via kisspeptin release from KNDy neurons (Weems *et al.* 2018). Thus, an E_2-induced decrease in NKB would be expected to act within the KNDy network to decrease kisspeptin release and subsequently GnRH pulse amplitude. In conclusion, it appears that KNDy neurons mediate the negative feedback actions of both progesterone and E_2 during the breeding season, with dynorphin and kisspeptin, respectively, being the primary outputs producing these effects.

Effects of external inputs on GnRH pulsatility in adults

Three external factors can clearly inhibit pulsatile GnRH secretion in adult ewes: stressors, undernutrition, and photoperiod. While it is well known that stress can inhibit GnRH secretion in a number of species including sheep, the underlying neuroendocrine mechanisms are complex and not completely understood. The effects of a variety of different stressors on pulsatile LH secretion have been examined in ewes, ranging in severity (based on cortisol concentrations produced by each stressor) from mild (psychosocial and transport stress) through moderate (insulin-induced hypoglycemia) to severe (endotoxin). Although there is little direct information on pulsatile GnRH secretion, all four stressors likely inhibit GnRH pulse frequency based on their ability to inhibit LH pulse frequency (Goodman & Inskeep 2006). However, measurements of GnRH pulses demonstrated that endotoxin

(Battaglia *et al.* 1997) and psychosocial stress (Wagenmaker *et al.* 2009) inhibit GnRH pulse amplitude, but had less consistent effects on GnRH pulse frequency; psychosocial stress had no consistent effect on pulse frequency, while endotoxin inhibited pulsatile GnRH secretion, but did not significantly decrease pulse frequency because of variability in the timing of this inhibition. It should be noted that these experiments were done using OVX ewes so the results do not rule out a stronger inhibition of GnRH pulsatility in ovary-intact animals (see below).

There has been considerable work on the role of increased cortisol concentrations in the stress-induced inhibition of episodic GnRH/LH secretion that has revealed a major difference in the actions of this glucocorticoid depending on the presence or absence of ovarian steroids (Ralph *et al.* 2016). In OVX ewes, cortisol acts solely at the pituitary via the Type II glucocorticoid receptor (GR) to inhibit the response to GnRH, and thus blunts LH pulse amplitude, without altering LH pulse frequency or episodic GnRH secretion (Breen & Karsch 2004). In contrast, in follicular phase animals or in OVX ewes given exogenous steroids to mimic a follicular phase, cortisol inhibits GnRH (Oakley *et al.* 2009a) and LH (Breen *et al.* 2005, Oakley *et al.* 2009a, Oakley *et al.* 2009b) pulse frequency. Cortisol produced no consistent effects on GnRH pulse amplitude (Oakley *et al.* 2009a) with a modest inhibition of LH pulse amplitude seen in some, but not all, studies (Breen *et al.* 2005, Oakley *et al.* 2009a, Oakley *et al.* 2009b). There is one report that the inhibition of GnRH pulse amplitude by psychosocial stress was not affected by the GR (and PR) antagonist, RU486 (Wagenmaker *et al.* 2009), but this experiment used OVX ewes, so its relevance to normal animals remains unclear. Thus, in ovary-intact ewes cortisol produces a significant decrease in GnRH pulse frequency that likely contributes to the inhibitory effects of stress.

There is essentially no published data on the role of KNDy neurons in the inhibitory response to cortisol in ewes, but we have preliminary evidence that dynorphin release from KNDy neurons in the middle ARC may play a role. This sub-population of KNDy neurons, but not KNDy neurons in the caudal or rostral ARC, contains GR and exogenous cortisol increases preprodynorphin mRNA and dynorphin protein expression in this KNDy subpopulation (Ralph *et al.* 2016). If these data are confirmed they will provide another important function for KNDy neurons in the control of episodic GnRH secretion. Finally it is important to note that cortisol, by itself, cannot account for the inhibition of episodic GnRH or LH secretion by stressors (Debus *et al.* 2002, Wagenmaker *et al.* 2009), so neural systems activated (or inhibited) by these stressors must play some role. However, the systems involved remain largely unknown because the most likely candidates (corticotropin releasing hormone or arginine vasopressin) do not inhibit, and in some cases actually stimulate, LH secretion in ewes (Goodman & Inskeep 2006). Recent work has focused on a possible role for RFRP-3 neurons because stronger stressors, such as insulin or endotoxin, increase Fos expression in RFRP-3 neurons and the percentage of GnRH cells with close contacts containing RFRP-3 (Papargiris *et al.* 2011, Clarke *et al.* 2016). However, the functional significance of these changes awaits studies determining if antagonists to the RFRP-3 receptor can block the stress-induced inhibition of GnRH or LH.

As is the case for pre-pubertal ewe lambs, undernutrition in adult ewes reduces LH secretion. However, in the adult ewe a longer duration of food restriction is required to produce a reduction in LH (e.g. pre-pubertal: 2 months (Wang *et al.* 2016); adult: 6 months (Backholer *et al.* 2010a)), which is thought to reflect the larger metabolic reserve (i.e., adipose) in the adult. One possible peripheral signal linking nutrition to reproduction is the adipose-derived hormone leptin. Circulating concentrations of leptin closely correlate with body fat (Delavaud *et al.* 2000, Daniel *et al.* 2002), are reduced during times of undernutrition (Daniel *et al.* 2002), and are associated with fertility and ovulation rate in sheep (Nieto *et al.* 2013). While there is little evidence for a central action of leptin on LH secretion in pre-pubertal ewes, central administration of leptin in chronically food restricted adult ewes stimulates LH secretion (Henry *et al.* 2001, Backholer *et al.* 2010a). Given that GnRH neurons in other species appear to be devoid of leptin receptors (Finn *et al.* 1998, Hakansson *et al.* 1998), the stimulatory action of centrally administered leptin is likely upstream of these key reproductive neurons.

In sheep, there is currently limited data as to whether KNDy neurons are part of the central mechanisms whereby undernutrition inhibits GnRH/LH release. It has been shown that chronic food restriction (6–10 months) reduced kisspeptin mRNA expression in the POA and ARC of OVX ewes, an effect that was partially restored with a sustained central infusion of leptin (Backholer *et al.* 2010b). Initially KNDy neurons were thought to be direct targets of leptin because essentially all ARC kisspeptin neurons express leptin receptors (Backholer *et al.* 2010b). However, this concept has since been challenged by others who demonstrated that leptin administration fails to activate ARC kisspeptin neurons as assessed with p-STAT3-ir (Louis *et al.* 2011). Interestingly, KNDy neurons may serve as an intermediary for classic energy sensing neurons such as proopiomelanocortin (POMC) and NPY/agouti-related peptide (AgRP) neurons, both of which are known to influence reproduction in several species. In adult ewes, ARC kisspeptin neurons receive afferent input from both POMC and NPY neurons (Backholer *et al.* 2010b), but the effect of energy balance on this connection remains to be determined. Furthermore, central infusion of melanotan II, an agonist for α-MSH receptors, stimulated LH secretion in fed (Backholer *et al.* 2009) and food restricted adult ewes (Backholer *et al.* 2010a), but the specific role of KNDy neurons in the actions of POMC and/or NPY/AgRP neurons to control GnRH/LH secretion remains largely unknown.

In contrast to stress and nutrition, the effects of photoperiod on reproduction have been extensively investigated, and there is thus considerable information on the neural systems involved in the photoperiodic control of GnRH pulses in ewes. In most breeds of sheep, ewes have annual fluctuations in fertility, with ovulatory cycles occurring in the fall and winter months (breeding season) and no ovulations during the spring and summer months (anoestrous season). The timing of these fluctuations is controlled by the external photoperiod (hours of light/day) and ensures that lambs are born in the spring when environmental conditions favor their survival (Hazlerigg & Simonneaux 2015). Studies of seasonal breeding fall into two major categories: 1) the mechanisms by which changes in photoperiod are perceived and 2) seasonal changes in the hypothalamo-hypophysial unit that produce fertility and infertility. The former, which involves photoperiod-induced changes in melatonin secretion from the pineal gland and its actions in the pars tuberalis is beyond the

scope of this review; for interested readers, details can be found in several recent reviews (Goodman *et al.* 2010, Hazlerigg & Simonneaux 2015, Weems *et al.* 2015)). Instead, we will focus on the latter, which involves changes in pulsatile GnRH secretion.

Early studies on the neuroendocrine mechanisms responsible for the seasonal changes in fertility in ewes found that: 1) there is a marked increase in response to the negative feedback actions of E_2 in the anoestrous season (Legan *et al.* 1977), 2) this increase reflects a change in the effects of E_2 on episodic GnRH secretion; during the breeding season, E_2 inhibits GnRH pulse amplitude while during anoestrus it strongly inhibits GnRH pulse frequency (Goodman *et al.* 1982), and 3) there is a modest decrease in GnRH pulse frequency in anoestrous OVX ewes (known as the steroid-independent effects of photoperiod), that is accompanied by an increase in pulse amplitude (Goodman *et al.* 1982).

Much of the work on seasonal breeding after these initial observations, and before the discovery of kisspeptin, focused on the neural mechanisms underlying the shifts in the ability of E_2 to inhibit GnRH pulse frequency (Goodman *et al.* 2010). Briefly, the model that developed from this work proposed that a group of inhibitory dopaminergic (DA) neurons in the retrochiasmatic area of the ovine hypothalamus, known as A15 neurons (Thiery *et al.* 1989, Thiery *et al.* 1995), are only active in anoestus and mediate the negative feedback actions of E_2 on GnRH pulse frequency at this time of year (Havern *et al.* 1994, Lehman *et al.* 1996). A15 neurons do not contain ERα and are thought to receive oestrogen-responsive afferents (possibly GABAergic or glutamatergic neurons (Bogusz *et al.* 2008, Singh *et al.* 2009)) from the POA (Anderson *et al.* 2001) and retrochiasmatic area (Gallegos-Sanchez *et al.* 1997, Hardy *et al.* 2003) that stimulate these inhibitory neurons. This population of DA neurons project caudally to the MBH, not rostrally to the POA (Gayrard *et al.* 1995, Goodman *et al.* 2010), but the mechanisms by which A15 neurons inhibit GnRH/LH pulse frequency during anoestrus remained largely unknown until recently, when a model incorporating KNDy neurons was developed.

This model arose from the initial observation that there is a seasonal difference in the ability of E_2 to inhibit kisspeptin expression in the ARC (Smith *et al.* 2008). During the breeding season, E_2 decreases the number of kisspeptin-containing KNDy neurons, but this effect is greatly enhanced in anoestrus. Thus, there are many fewer kisspeptin neurons in the ARC (but not in the POA), of E_2-treated OVX ewes during anoestrus than during the breeding season. This seasonal difference is associated with a decrease in kisspeptin inputs to MBH, but not POA, GnRH neurons (Smith *et al.* 2008).

These data led to the hypothesis that A15 DA neurons act via inhibition of kisspeptin release to reduce GnRH/LH pulse frequency in anestrous ewes, which is now supported by several lines of evidence. First, most KNDy neurons contain the D2 dopamine receptor (Goodman *et al.* 2012, Weems *et al.* 2017a) that mediates the actions of DA on GnRH pulse frequency in anoestrus (Goodman *et al.* 2010). Second, E_2 increases both the percentage of KNDy neurons containing the D2 receptor and those receiving DA-containing close contacts in anoestrous ewes (Weems *et al.* 2017a). Finally, functional evidence for this connection includes the ability of microinjections of the D2 receptor antagonist, pimozide, into the ARC to increase LH pulse frequency in anoestrus and that the stimulatory actions of an im

injection of pimozide were blocked by icv infusions of the KISS1R antagonist, p-271(Goodman *et al.* 2012). Based on these data, it has been proposed that the increased ability of E_2 to inhibit GnRH pulse frequency in anoestrus reflects both increased release of DA from A15 projections onto KNDy neurons and an increased response of KNDy neurons to this inhibitory neurotransmitter.

Recent work has raised the possibility that two other neurotransmitters, RFamide-related peptide-3 (RFRP-3) and somatostatin, may also be involved in the seasonal regulation of episodic GnRH secretion. The number of RFRP-3-ir cell bodies in the paraventricular nucleus (PVN) and dorsomedial hypothalamus (DMH) increases during anoestrus in estrogen-treated OVX ewes (Smith *et al.* 2008), and RFRP-3 concentrations in hypophysial portal blood of ovary-intact ewes are higher in anoestrus than in the breeding season (Smith *et al.* 2012). The ISH data on expression of mRNA for RFRP-3 are inconsistent, with one report of a small (17%) increase in the PVN/DMH in ewes transferred from short to long days (Dardente *et al.* 2008) but another that found no effect of season in ewes kept on natural photoperiod (Smith *et al.* 2008). Studies on the possible actions of RFRP-3 in ewes have focused mainly on potential effects at the pituitary and there is both in vitro (Clarke *et al.* 2008, Sari *et al.* 2009) and in vivo (Clarke *et al.* 2012, Smith *et al.* 2012) evidence that RFRP-3 decreases GnRH-induced LH secretion. However, the in vivo studies used doses of RFRP-3 that produced concentrations of this peptide in the hypophysial portal circulation many times greater than those in normal animals (Smith *et al.* 2012), and these results were not confirmed by another group (Caraty *et al.* 2012, Decourt *et al.* 2016). There may be important seasonal effects of RFRP-3 on GnRH secretion because RFRP-3 input to GnRH neurons increases in anestrus (Smith *et al.* 2008). However, expression of the RFRP-3 receptor was not found in areas containing GnRH cell bodies (Dardente *et al.* 2008) and administration of either RFRP-3 (Caraty *et al.* 2012) or a specific antagonist to the RFRP-3 receptor (Decourt *et al.* 2016) into the third ventricle had no effect on LH secretion. Thus any conclusions on the role of RFRP-3 in seasonal changes in pulsatile secretion of GnRH/LH in ewes awaits further work to resolve the contradictory data currently available.

There is stronger evidence that somatostatin contributes to the steroid-independent actions of inhibitory photoperiod. Specifically, icv injections of a somatostatin receptor antagonist increased LH pulse frequency in OVX anestrous ewes to levels similar to those in OVX breeding season animals, but this antagonist had no effects in the latter (McCosh *et al.* 2017). A seasonal difference was also observed in ovary-intact animals with a 300% increase in mean LH concentrations in anoestrous animals compared to only a 40% increase in breeding season (luteal phase) ewes (McCosh *et al.* 2017). Interestingly, this somatostatin receptor antagonist also produced a modest increase in the percentage of kisspeptin neurons containing Fos in the caudal ARC in ovary-intact anestrous ewes. This limited activation of KNDy neurons could reflect the relatively modest changes in LH pulse frequency produced by the steroid-independent effects of photoperiod. Because early pharmacological data had implicated serotonin in the seasonal changes in LH pulse frequency in OVX ewes, it was proposed that serotonin acts via somatostatin interneurons to inhibit KNDy neurons.

Conclusion

Classic studies in the 1980s and 1990s provide a wealth of evidence on the control of pulsatile GnRH secretion by internal (i.e., ovarian steroids) and external (i.e stress, nutrition, and photoperiod) inputs in ewes. However, all these findings had to be interpreted in the context of the "GnRH pulse generator" being considered a "black box" because of the paucity of information on the neural mechanisms driving episodic GnRH secretion. The recent discovery of the reproductive actions of kisspeptin, and the subsequent development of the KNDy hypothesis for GnRH pulse generation, provide a basis for novel neural mechanisms by which these inputs control GnRH, and thus LH, pulses.

While the roles of ovine KNDy neurons in mediating the actions of these internal and external inputs have yet to be extensively studied, some useful conclusions can be drawn (Figure 5). The strongest of these are the mechanisms by which ovarian steroids inhibit LH secretion in adult ewes. During the breeding season there is strong evidence that progesterone inhibits GnRH pulse frequency via increased dynorphin release from KNDy neurons which likely acts within the KNDy network and directly on GnRH neurons. In contrast, during this season E_2 inhibits GnRH pulse amplitude by inhibiting kisspeptin release onto GnRH neurons, although a role for changes in NKB and/or dynorphin release onto other KNDy neurons cannot be ruled out. In adult anoestrous ewes, the inhibitory photoperiod activates A15 DA neurons which respond to an increase in E_2 by releasing DA onto KNDy neurons to inhibit GnRH pulse frequency.

There is relatively less information on the role of KNDy neurons in controlling episodic GnRH secretion prior to puberty and in response to stressors and undernutrition. Prior to puberty it is clear that E_2 inhibits GnRH pulse frequency. There is strong evidence that this reflects an inhibition of kisspeptin release from KNDy neurons, and stimulation of dynorphin release (which may come from KNDy neurons), that wanes with the decrease in the negative feedback actions of E_2 during puberty (Figure 5). There is some evidence that an increase in NKB may also be involved in puberty in ewe lambs, but this hypothesis requires further testing. Moreover, whether E_2 acts direct on KNDy neurons prior to puberty to inhibit GnRH pulse frequency or via neurons afferent to the KNDy network, as it does during anoestrus, remains to be determined. Even less is known about the role of KNDy neurons in the response to undernutrition and stress in ewes. Inadequate nutrition undoubtedly inhibits leptin concentrations which likely decrease KNDy neural activity possibly via POMC or AgRP neurons in both lambs and adult ewes. However, much of the data for this hypothesis relies on work in other species (Manfredi-Lozano *et al.* 2016, Padilla *et al.* 2017). Similarly, stressors clearly inhibit GnRH pulse frequency in ewes; this likely reflects both direct effects of cortisol on KNDy neural activity and the activation of unknown afferents that also inhibit the activity of the GnRH pulse generator. Finally, it is important to note that earlier work implicated many other neurotransmitters (e.g., glutamate, GABA, norepinephrine) in the control of episodic GnRH secretion in ewes (Goodman & Inskeep 2015); the possible interaction of some of these (e.g., glutamate (Goodman *et al.* 2013, Ezzat *et al.* 2015, Merkley *et al.* 2015)) with KNDy neurons has started to be explored, but more work is needed to integrate earlier and more recent data sets. In conclusion, the development of the KNDy hypothesis for GnRH pulse generation has also

set the stage for a more detailed understanding of the neural systems by which external and internal inputs modify pulsatile GnRH secretion in ewes. Although these investigations are in their early stages, this model can be used to develop specific hypotheses and predictions that can then be tested in future work.

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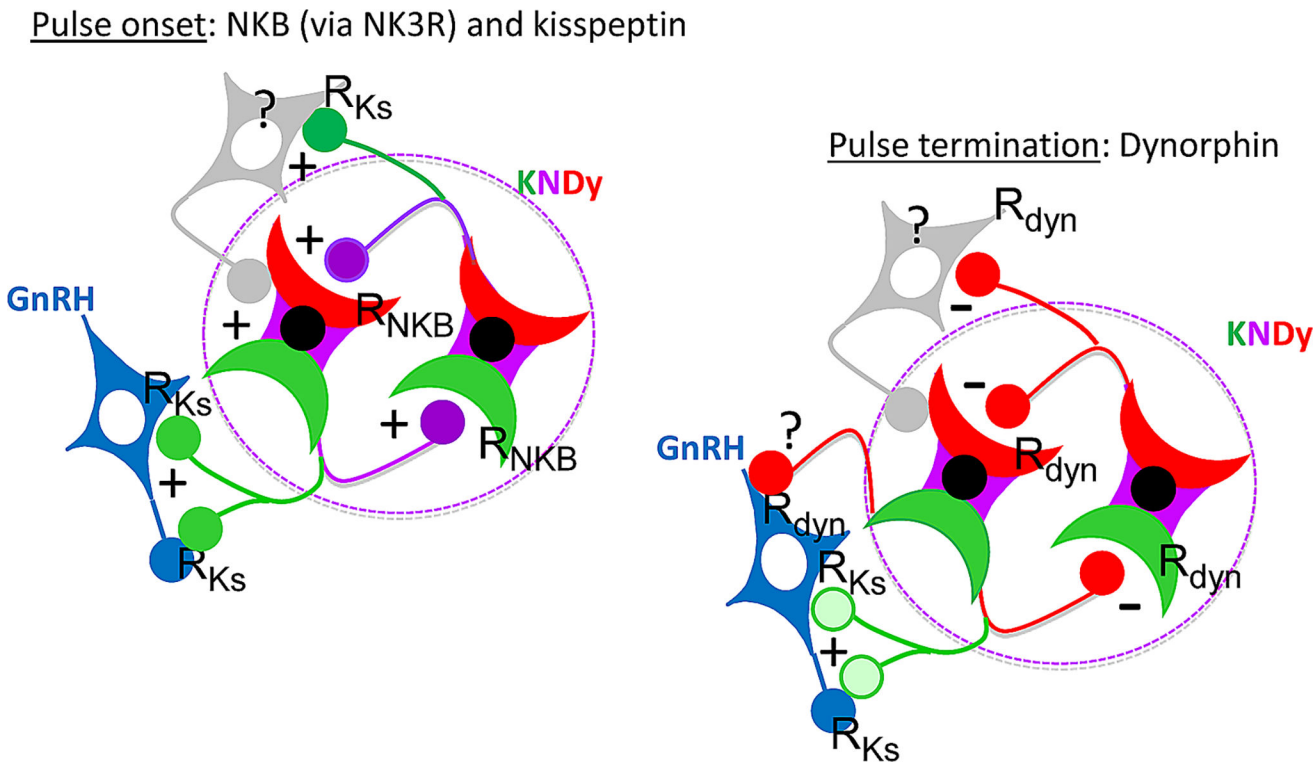

Figure 1.

Model for control of KNDy neural activity proposed to drive episodic GnRH secretion. Each GnRH pulse is initiated by NKB (purple) acting within the KNDy network (within dashed oval), which stimulates kisspeptin (green) release to drive GnRH (blue) secretion and activate unidentified Kiss1r-containing ARC neurons (grey) that reinforces the stimulatory actions of NKB on KNDy neurons. GnRH release is then terminated by dynorphin (red) release from KNDy neurons acting either directly on KNDy neurons and/or GnRH neurons and/or the unidentified Kiss1r-containing neurons. Abbreviations: R_{dyn}: kappa-opioid receptor; R_{Ks}: Kiss1r; R_{NKB}: NK3R. Note that the color in each terminal indicates the biologically active transmitter (possibly due to selective expression of post-synaptic receptors) and does not reflect selective transport of that peptide to the terminal. Redrawn from Lehman et al., (8) with permission from the Endocrine Society.

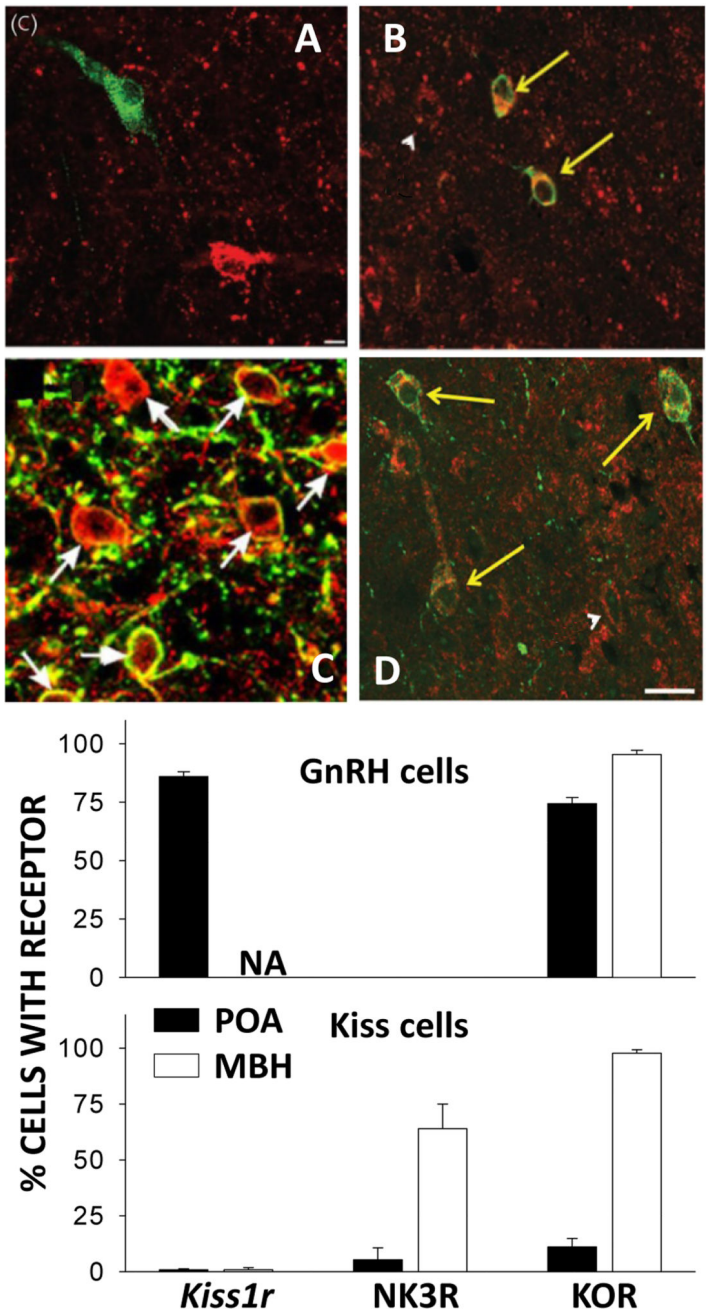

Figure 2.

Receptors for KNDy peptides in GnRH and kisspeptin neurons. Top four panels depict dual ICC for NK3R (red in A, green in C) and KOR (red in B and D) in GnRH neurons (green in A and B) and kisspeptin neurons (red in C, green in D). Arrows identify dual labeled cells, arrowheads indicate single labeled KOR neurons. Bottom panels present the mean (± SEM) percentage of GnRH and kisspeptin cells in the POA and MBH (KNDy cells for latter) containing each receptor. Note no GnRH cells contained NK3R. NA: Not analyzed. Photomicrographs in Panels A and C reprinted from Amstalden et al., 2010 and Ahn et al., J Neuroendocrinol 27: 100–110, 2015, respectively with permission from the British Society

for Neuroendocrinology. Photomicrographs in panels B and D reprinted from Weems et al. 2016, with permission from the Endocrine Society. Bar graphs in bottom panel are based on data from these three references (NK3R and KOR) and from Smith et al, 2011 (*Kiss1r*).

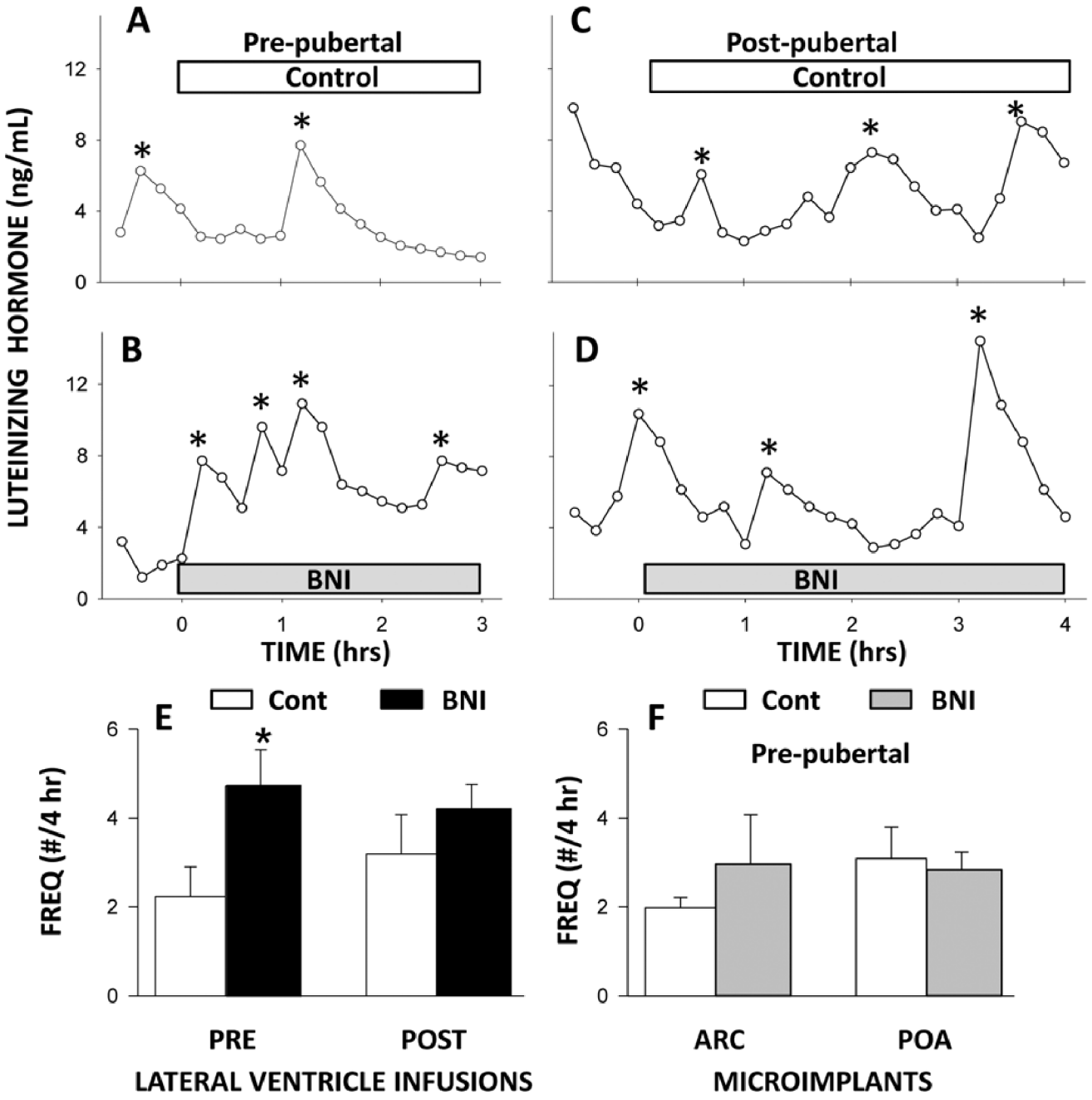

Figure 3.

Effect of a KOR antagonist on LH pulse frequency in pre- and post-pubertal lambs. Top panels: LH pulse patterns in response to lateral ventricle infusions of vehicle (Control) or a KOR antagonist (BNI) in OVX+E pre-pubertal (Panels A,B) and post-pubertal (Panels C,D) lambs. *Peak of LH pulse. Bottom panels: Mean (± SEM) LH pulse frequency in response to lateral ventricle infusions of vehicle (Cont) or the KOR antagonist (BNI) in oestrogen-treated OVX pre- and post-pubertal lambs are shown on left (Panel E). Effect of empty (Cont) or BNI-filled microimplants in the ARC or POA of ovary-intact pre-pubertal lambs on pulse frequency are depicted on right (Panel F).*P<0.05 vs Cont. Data on lateral ventricle

infusions redrawn from Lopez et al., 2016 with permission from the British Society for Neuroendocrinology. Data on the effects of microimplants have not been published.

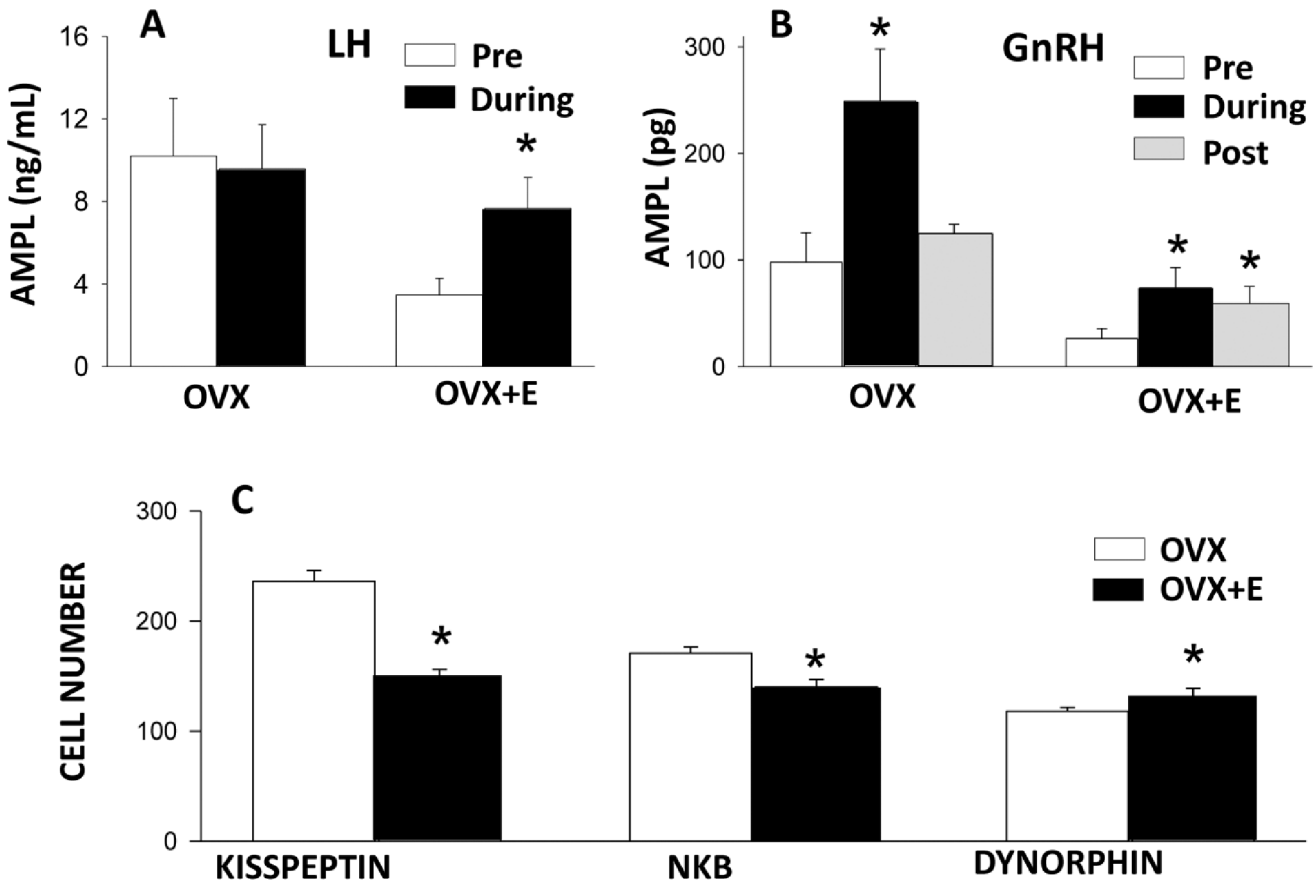

Figure 4.

Evidence for possible roles of each KNDy peptide in mediating E_2 negative feedback in breeding season ewes. Top panels: Effect of an EOP receptor antagonist on mean (± SEM) LH (A) and GnRH (B) pulse amplitude in OVX and OVX+E breeding season ewes. * P<0.05 vs pre-treatment values. Bottom panel (C): Effect of E_2 on the mean (± SEM) number of cells in the ARC containing kisspeptin, NKB, or dynorphin in OVX breeding season ewes. *P<0.05 vs OVX. LH data reprinted from Whisnant and Goodman, Biol Reprod 39: 1032–38, 1988 with permission from the Society for the Study of Reproduction. GnRH and KNDy peptide data reprinted from Goodman et al., 1995 and Weems et al., 2017a, respectively, with permission of the Endocrine Society.

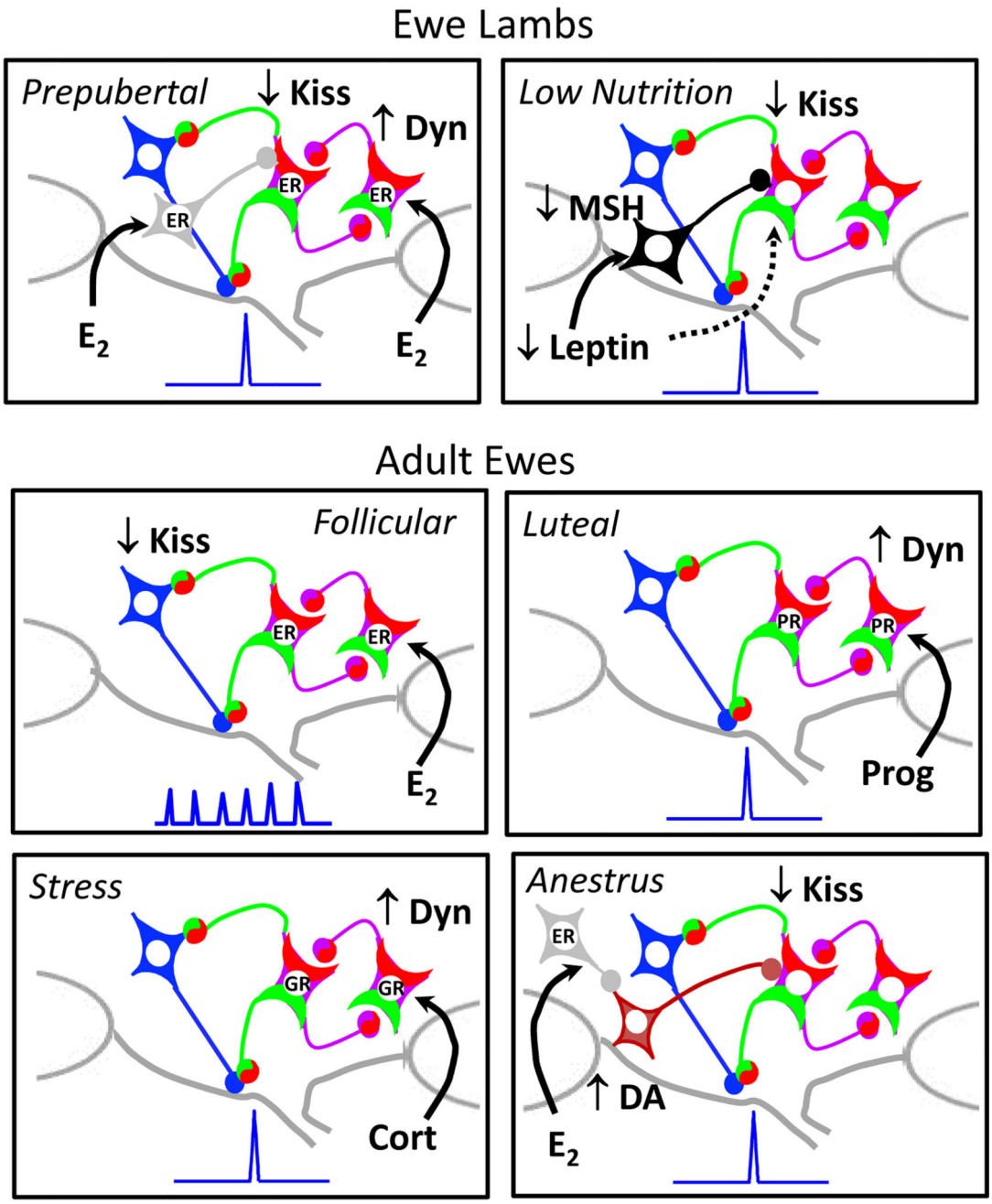

Figure 5.

Role of KNDy neurons in the control of pulsatile GnRH secretion in ewes under different physiological conditions. In well-fed prepubertal lambs (top left panel), GnRH pulse frequency is held in check by E_2-induced decreases in kisspeptin and increases in dynorphin release, but this could be due to either direct or indirect actions of E_2 on KNDy neurons. In lambs (and adult ewes) on a low level of nutrition (top right panel), a fall in leptin suppresses GnRH release by inhibiting kisspeptin via MSH; it is unclear if leptin also has direct effects on KNDy neurons. In breeding season adults (middle panels), E_2 acts during the follicular phase to decrease kisspeptin release and thus GnRH pulse amplitude and progesterone acts

during the luteal phase to inhibit pulse frequency via an increase in dynorphin. In follicular phase ewes, stressors inhibit GnRH pulse frequency via cortisol, which may act to increase dynorphin output from KNDy neurons (bottom left panel) and unidentified neural inputs (not illustrated). In anoestrous ewes (bottom right panel) E_2 acts via afferent input to increase DA release from A15 neurons that innervate KNDy neurons and inhibit kisspeptin and thus GnRH pulse frequency.