REVIEW

Kisspeptin and the regulation of the reproductive axis in domestic animals

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Abstract

The control of reproductive processes involves the integration of a number of factors from the internal and external environment, with the final output signal of these processes being the pulsatile secretion of gonadotrophin-releasing hormone (GnRH) from the hypothalamus. These factors include the feedback actions of sex steroids, feed intake and nutritional status, season/photoperiod, pheromones, age and stress. Understanding these factors and how they influence GnRH secretion and hence reproduction is important for the management of farm animals. There is evidence that the RF-amide neuropeptide, kisspeptin, may be involved in relaying the effects of these factors to the GnRH neurons. This paper will review the evidence from the common domestic animals (sheep, goats, cattle, horses and pigs), that kisspeptin neurons are (i) regulated by the factors listed above, (ii) contact GnRH neurons and (iii) involved in the regulation of GnRH/gonadotrophin secretion.

Key Words

- kisspeptin
- GnRH
- hypothalamus
- domestic animals

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Introduction

Brain control of reproduction in mammals involves the integration of a number of factors. In females, these factors impact the ability to attain pregnancy and carry the fetus to term, as well as the ability to suckle the offspring post-partum. Many of these factors also influence reproduction in males. They include gonadal status, as signalled by gonadal hormone secretion, age and body condition of the animals, stressors, pheromones, body rhythms (ultradian, circadian, infradian and circannual) and, in some animals, season/photoperiod. The final output signal of this integration is the release into hypophyseal portal blood of the neurohormone gonadotrophin-releasing hormone (GnRH). Anterior pituitary secretion of the gonadotrophin, luteinising hormone (LH), then acts as an amplifier to the GnRH signal (Clarke & Cummins 1982). In sheep, at least, the neurons that produce GnRH receive relatively little synaptic input compared to other neurons within the hypothalamus (Lehman *et al*. 1988, Magee *et al*. 2009, Decourt *et al*. 2014, McGrath *et al*. 2016). Thus, it would seem likely that much of the integration of the various factors that influence GnRH secretion occurs upstream of the GnRH neuron. There is substantial evidence for a wide array of different neurons that regulate GnRH activity, but one particular set of neurons has come to prominence, those that produce kisspeptin. It is a potent regulator of GnRH and LH secretion, while there is evidence that all of the factors listed above can influence kisspeptin.

The sheep has proven to be an excellent model for neuroendocrine research and a great deal of research into the regulation and actions of kisspeptin has been conducted using this model. It is clear that in animal production

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systems, reproductive success is critical to the operation of these enterprises, hence, a good understanding of the control of GnRH is necessary. Accordingly, in recent times, this research has spread to other major domestic animal species, especially goats, cattle, horses and pigs, where commonalities have been observed, as well as significant species differences. This review will examine the current state of knowledge regarding kisspeptin neurons in these five species. Firstly, we will examine these neurons and their regulation, then the evidence for their interaction with GnRH neurons, and finally their action on GnRH and/or LH secretion.

Location of Kisspeptin neurons

The anatomy of the kisspeptin neural network in mammals has been thoroughly reviewed previously (Lehman *et al*. 2010*b*). The main population of kisspeptin neurons is in the arcuate nucleus of the hypothalamus, especially in the more caudal region of the nucleus, extending to around the pre-mammillary nucleus. This is well conserved (Table 1), being the case in sheep (Estrada *et al*. 2006, Franceschini *et al*. 2006, Goodman *et al*. 2007), goats (Okamura *et al*. 2017), cattle (Hassaneen *et al*. 2016, Tanco *et al*. 2016), horses (Decourt *et al*. 2008, Magee *et al*. 2009) and pigs (Tomikawa *et al*. 2010).

The second largest population of kisspeptin cells, albeit much smaller in number, is found in the medial preoptic area, in sheep, goats and cattle (Estrada *et al*. 2006, Franceschini *et al*. 2006, Goodman *et al*. 2007, Matsuda *et al*. 2015, Hassaneen *et al*. 2016, Tanco *et al*. 2016). Horses (Decourt *et al*. 2008, McGrath 2015), by contrast, do not appear to contain kisspeptin neurons in the preoptic area at all. In pigs, the rostral population of kisspeptin neurons is in the periventricular nucleus rather than the preoptic area (Tomikawa *et al*. 2010). Some cells have also been described in the dorsomedial nucleus and a small number in the ventromedial nucleus in sheep (Franceschini *et al*. 2006), horses (Decourt *et al*. 2008) and cattle (Hassaneen *et al*. 2016).

Nearly all the kisspeptin neurons in the arcuate nucleus co-express neurokinin B and dynorphin (Goodman *et al*. 2007, Wakabayashi *et al*. 2010, Hassaneen *et al*. 2016). They have been branded KNDy (kisspeptin/neurokinin B/dynorphin) neurons, based on this co-localisation. KNDy neurons have been observed only in the arcuate population and not in other kisspeptin neurons. They form an interactive network (Foradori *et al*. 2006) and the various roles for kisspeptin, neurokinin B and dynorphin appear critical for their function, as described in detail later. Tract tracing studies in goats (Wakabayashi *et al*. 2013) have shown extensive communication between the left and right hand sides of the nucleus, mainly via neurokinin B. In sheep (Amstalden *et al*. 2010) and goats (Wakabayashi *et al*. 2010), the majority of KNDy neurons express the NK3 receptor, the receptor for neurokinin B, while around 90% of KNDy neurons in sheep express the kappa opioid receptor, the receptor for dynorphin (Weems *et al*. 2016). Thus, the KNDy neurons express both the NK3 and kappa opioid receptors. By contrast, kisspeptin neurons do not express the kisspeptin receptor (kiss1R) (Smith *et al*. 2011), indicating that communication between the KNDy neurons is via neurokinin B and dynorphin but not by kisspeptin. This connection appears to be functional, because central infusion of neurokinin B in anoestrous ewes activated kisspeptin neurons, with a substantial increase in the percentage of kisspeptin neurons expressing the Fos protein, a marker of neuronal activation (Sakamoto *et al*. 2012). Finally, these KNDy neurons also release glutamate, as evidenced by the presence of the vesicular glutamate transporter protein, vGlut2, in the terminals of KNDy neurons (Merkley *et al*. 2015).

The distribution of kisspeptin fibres has been described in all the species under consideration (Franceschini *et al*. 2006, Decourt *et al*. 2015, Hassaneen *et al*. 2016, Okamura *et al*. 2017), except pigs. The distribution appears to be well conserved across species. Notable is a collection of immunoreactive fibres in the external zone of the median eminence, indicating that the kisspeptin peptide is secreted into the portal

Table 1 The location of the main kisspeptin neuronal populations in the brain of domestic animal species.

Species	Region	References
Sheep	ARC, mPOA	Estrada *et al*. (2006), Franceschini *et al*. (2006), Goodman *et al*. (2007)
Horse	ARC	Decourt *et al*. (2008), McGrath (2015)
Cattle	ARC, mPOA	Hassaneen *et al*. (2016), Tanco *et al*. (2016)
Goat	ARC, mPOA	Okamura *et al*. (2017)
Pig	ARC, PVN	Tomikawa *et al*. (2010)

ARC, arcuate nucleus; mPOA, medial pre-optic area; PVN, para-ventricular nucleus.

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vasculature (Smith 2008). Similarly, in all the species there are abundant fibres in the internal zone, allowing for interaction with fibres of neurons that also secrete into portal vessels (Ohkura *et al.* 2009*b*). The arcuate nucleus contains abundant kisspeptin fibres, especially surrounding kisspeptin somata. Kisspeptin fibres are found throughout the septo-preoptic region, which is the region that contains the majority of GnRH neurons (Lehman *et al.* 1986). Other larger fibre populations include a population running adjacent to, and parallel to the third ventricle.

The kisspeptin fibres found in the median eminence all appear to be of KNDy origin (Smith *et al.* 2011), indicating that they originate in the arcuate nucleus and not from the other hypothalamic regions. This is confirmed by anterograde tracing studies in ewes, which indicate projections of kisspeptin neurons from the arcuate nucleus to the median eminence (Smith *et al.* 2011). Similarly, most kisspeptin inputs to the preoptic kisspeptin neurons are also from KNDy neurons (Smith *et al.* 2011, Merkley *et al.* 2015).

In sheep, a sex difference has been observed in the kisspeptin neuronal populations. This was first observed for neurokinin B neurons, long before KNDy neurons were discovered (Goubillon *et al.* 2000), where rams showed fewer neurokinin B immunoreactive (-ir) cells within the caudal arcuate nucleus than observed in ewes. This was subsequently confirmed for KNDy neurons, where rams had nearly half the number of kisspeptin, neurokinin B and dynorphin-positive cells in the arcuate nucleus as did ewes (Cheng *et al.* 2010). This difference is manifest primarily in the most caudal parts of the arcuate nucleus (Goubillon *et al.* 2000, Cheng *et al.* 2010), with little difference in cell numbers in the rostral arcuate. Similarly, significantly higher numbers of kisspeptin-ir neurons were found in the preoptic area of ewes compared with rams (Cheng *et al.* 2010). Cernea *et al.* (2015) showed that the sex difference in the KNDy population is most likely due, at least in part, to an organisational action of testosterone, since prenatal treatment of ewe lambs with testosterone resulted in smaller KNDy cells and a reduction in the number of synaptic inputs to KNDy cells, especially KNDy to KNDy connections, as well as the number of inputs to GnRH neurons. This treatment did not reduce the number of kisspeptin cells, however, indicating that testosterone action during development is not the sole reason for the sex difference in the KNDy neuronal population (Cheng *et al.* 2010).

Regulation of Kisspeptin neurons

Connections to kisspeptin neurons

The identity of the neuronal inputs to kisspeptin neurons in domestic animals is summarised in Table 2. Most of what is known has been derived from data in sheep. From immunohistochemical studies, there is evidence of synaptic input to kisspeptin neurons from neurons that produce glutamate (Merkley *et al.* 2015), dopamine (Goodman *et al.* 2012), neuropeptide Y (NPY) (Backholer *et al.* 2010), pro-opiomelanocortin (POMC) (Backholer *et al.* 2010), melanocyte-stimulating hormone (Cardoso *et al.* 2015), GnRH (Rose 2017) as well as dynorphin (Weems *et al.* 2015), neurokinin B (Amstalden *et al.* 2010, Wakabayashi *et al.* 2013) and kisspeptin (Goodman *et al.* 2007). These latter three form the interconnecting network of KNDy neurons mentioned previously. Tract tracing studies show no projections from the rostral preoptic area to the arcuate nucleus (Backholer *et al.* 2010), indicating no inputs to KNDy neurons from the preoptic population of kisspeptin neurons, a result supported by the work of (Merkley *et al.* 2015), who showed that virtually all kisspeptin input to KNDy neurons is KNDy.

Table 2 Observed inputs to kisspeptin neurons in domestic species.

Input	Preoptic kisspeptin cells	Arcuate kisspeptin cells	Species	Reference
Neurokinin B	X	✓	Sheep Goat	Amstalden *et al.* (2010), Wakabayashi *et al.* (2013)
Dynorphin	X	✓	Sheep	Weems *et al.* (2016)
Glutamate	✓	✓	Sheep	Merkley *et al.* (2015)
GnRH	X	✓	Sheep	Rose (2017)
Dopamine	X	✓	Sheep	Goodman *et al.* (2012), Weems *et al.* (2017)
Neuropeptide Y	–	✓	Sheep	Backholer *et al.* (2010)
Pro-opiomelanocortin	–	✓	Sheep Cattle	Backholer *et al.* (2010), Cardoso *et al.* (2015)
Substance P	X	✓	Goat	Okamura *et al.* (2017)

✓, observed; X, not observed; –, not tested.

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Kisspeptin regulation by sex steroids

There is abundant evidence that kisspeptin neurons are regulated by sex steroids. The first evidence for this regulation came from studies of neurokinin B prior to the discovery of kisspeptin. An acute treatment of ovariectomised (OVX) ewes with oestrogen resulted in a reduction in neurokinin B mRNA levels (Pillon *et al.* 2003). More recent work, conducted primarily in sheep, has provided evidence that kisspeptin neurons are regulated by oestrogen, but in an inhibitory manner by low doses of oestrogen, stimulated by high doses of oestrogen, as well as inhibited by progesterone or testosterone. These are discussed separately below.

Negative feedback actions of oestrogen

Ovariectomy in ewes resulted in a substantial increase in mRNA expression of the gene for kisspeptin, *KISS1*, in the arcuate nucleus but not preoptic area (Smith *et al.* 2007), which was blocked by chronic oestrogen treatment. Similarly, ovariectomy in ewes during the non-breeding season resulted in greater numbers of kisspeptin-ir cells in the arcuate nucleus (but not preoptic area), as well as higher levels of Fos expression within arcuate kisspeptin neurons than in ovary intact ewes. This indicates that ovarian hormones (presumably oestrogen) were inhibiting kisspeptin neurons and their activation (Merkley *et al.* 2012). Similarly, treatment of adult OVX ewes (Smith *et al.* 2009*b*) and ewe lambs (Lopez *et al.* 2016) with an oestrogen implant during the breeding season resulted in reduced numbers of kisspeptin-ir cells in the arcuate nucleus that expressed Fos protein. Collectively, these results indicate that doses of oestrogen that normally exert a negative feedback action on GnRH secretion inhibit arcuate kisspeptin neurons.

Within KNDy neurons, the nature of this regulation varies between the various neuropeptides produced, with ovariectomy stimulating kisspeptin (Smith *et al.* 2007), and neurokinin B (Nestor *et al.* 2012) but inhibiting dynorphin (Foradori *et al.* 2006) expression. This variability is consistent with the proposed roles of each of these co-localised neuropeptides, as discussed later in this paper. Based on this evidence, though, it would seem likely that sex steroids must change the degree of co-localisation of kisspeptin, neurokinin B and dynorphin (and glutamate) within KNDy neurons (Goodman *et al.* 2013), but this hypothesis has not been directly tested.

Positive feedback actions of oestrogen

KISS1 mRNA levels in the arcuate nucleus were elevated during the late follicular phase of the oestrous cycle in the ewe compared with other phases of the cycle (Estrada *et al.* 2006). This is a time in which circulating oestrogen levels are normally high. This was paralleled by a similar increase in neurokinin B mRNA in mid arcuate kisspeptin neurons at the same stage (Li *et al.* 2015). Treatment of OVX ewes with an IM injection of oestradiol benzoate, at a dose that reliably induces an LH surge, produced an increase in Fos expression in arcuate kisspeptin neurons, indicating neuronal activation at that time (Smith *et al.* 2009*a*). The same study also reported an increase in *KISS1* mRNA in arcuate nucleus tissue collected from the late follicular phase of the cycle, compared with the luteal phase. In addition, the number of synaptic inputs to kisspeptin neurons in the arcuate nucleus was higher in oestrous ewes (a time of high circulating oestrogen), compared with ewes in the luteal phase (Merkley *et al.* 2015). These results suggest that, unlike female rodents, where the evidence strongly points to the arcuate kisspeptin population being negatively regulated by oestrogen and the rostral population being stimulated by higher levels of oestrogen (Smith *et al.* 2005), the arcuate population is regulated in both a positive and negative manner depending on the circumstances.

The regulation of the kisspeptin neurons at the time of oestrogen positive feedback is less clear. The oestradiol benzoate injection that induced Fos expression in arcuate kisspeptin neurons (*vide supra*; Smith *et al.* 2009*a*) had no effect on the expression of Fos in preoptic kisspeptin neurons. Similarly, the number of kisspeptin-ir neurons in the preoptic area in ewes from the late follicular phase was not different to those during the luteal phase (Smith *et al.* 2009*a*), nor was there a difference in the expression of Fos protein in those ewes. In contrast to these studies, however, is a study by Hoffman *et al.* (2011), which found an increase in Fos expression in the preoptic population of kisspeptin neurons at the start of the preovulatory LH surge in the ewe. Merkley *et al.* (2015) found an increase in the percentage of preoptic kisspeptin neurons expressing Fos at the time of the LH surge in the ewe. Similarly, *KISS1* mRNA expression in the preoptic area also increased in the late follicular phase (Smith *et al.* 2009*a*), presumably stimulated by the rising levels of circulating oestrogen. The differences in these studies may reflect the timing of tissue collection, with the negative results coming from those studies where the tissue was collected just prior to the commencement of the LH surge, whilst those studies

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reporting a response to oestrogen used tissue collected after the commencement of the LH surge. Thus, it seems most likely that acute oestrogen positive feedback stimulates preoptic kisspeptin neurons. Nonetheless, more work is needed to clarify the regulation of the preoptic kisspeptin neurons by oestrogen.

In pigs, a reduction in kisspeptin-ir cell number in the arcuate nucleus was observed in brain tissue collected 48 h after a high dose of oestrogen (around the time of the start of an oestrogen –induced LH surge), but an increase in cell number in the periventricular region (Tomikawa *et al*. 2010). Similarly, in OVX goats (and castrated males goats), intravenous infusion of oestrogen increased the expression of Fos protein within kisspeptin neurons in the preoptic area but had no effect in the arcuate nucleus (Matsuda *et al*. 2015). This suggests that the oestrogen regulation of kisspeptin in the pig and goat is more like that of the rat and mouse than in the sheep.

Progesterone

While much attention has been directed to the regulation of kisspeptin by oestrogen, little attention has been given to the regulation by progesterone. In one sheep study, OVX ewes treated with progesterone had moderately lower *KISS1* mRNA levels compared to OVX animals, which was a substantially smaller effect compared with oestrogen treatment (Smith *et al*. 2007). Progesterone treatment of OVX ewes had no effect on pre-prodynorphin mRNA expression in the arcuate nucleus (Foradori *et al*. 2005), which we now know would have included KNDy neurons. These results need to be interpreted with caution, however, because oestrogen is needed to upregulate progesterone receptors in the arcuate nucleus (Scott *et al*. 2000*a*) and so there may have been insufficient progesterone receptors to allow progesterone to have much of an effect. Thus, the evidence regarding progesterone action on KNDy neurons in sheep is poor. Two studies in cattle (Hassaneen *et al*. 2016, Tanco *et al*. 2016) compared kisspeptin cell numbers between cows in the luteal and late follicular phases. Both found higher numbers in the follicular phase than luteal phase, but Hassaneen *et al*. (2016) found this difference in the preoptic kisspeptin population, while Tanco *et al*. (2016) found their difference in the arcuate kisspeptin cells. The study by Hassaneen *et al*. (2016) observed differences in both circulating oestrogen and progesterone levels, with the authors considering that the difference is most likely to be due to oestrogen. By contrast, the study by Tanco *et al*. (2016) found that circulating oestrogen levels were similar in both groups, while there was a significant difference in progesterone levels, suggesting that it is progesterone having the main action, to inhibit kisspeptin expression in the arcuate nucleus. Preoptic kisspeptin expression was low in that study and did not differ between stages of the oestrous cycle.

Testicular hormones

There has been little work into the sex steroid regulation of kisspeptin in males of domestic animals. Very few kisspeptin-ir cells could be detected in intact rams (Nestor *et al*. 2012, Rose 2017) or bucks (Matsuyama *et al*. 2011), but large numbers of kisspeptin-ir cells were observed in the caudal arcuate nucleus in males that had been castrated (Matsuyama *et al*. 2011, Nestor *et al*. 2012, Rose 2017). Thus, testosterone appears to exert a very strong inhibitory action on kisspeptin neurons in male sheep.

Steroid receptors

Studies in sheep using intracranial implants of sex steroids (Blache *et al*. 1991, 1996, 1997, Scott *et al*. 1997, Caraty *et al*. 1998) indicate that in both males and females, the arcuate nucleus, and to a lesser extent the preoptic area are key sites for the actions of sex steroids in the hypothalamus. Thus, the sex steroids may act directly on kisspeptin neurons. In the ewe, around half of preoptic kisspeptin neurons expressed oestrogen receptor alpha (ERα), but virtually all kisspeptin neurons in the arcuate nucleus expressed ERα (Goubillon *et al*. 2000, Franceschini *et al*. 2006), progesterone receptors (Foradori *et al*. 2002, Dufourny *et al*. 2005, Smith *et al*. 2007) and/or androgen receptors (Rose 2017). Arcuate kisspeptin neurons in rams also express ERα and AR (Rose 2017). We also have preliminary evidence for the expression of ERα in the kisspeptin neurons of cattle (Rose *et al*. 2018). The situation in the horse, goat and pig is unknown.

The expression of oestrogen receptor beta (ERβ) within kisspeptin neurons has not been studied in domestic animals. Since there is no ERβ in the arcuate nucleus of the sheep (Scott *et al*. 2000*b*), ERβ is unlikely to be expressed by KNDy neurons. There is, however, abundant ERβ expression in the preoptic area and so expression within preoptic kisspeptin neurons is possible. Since there is little evidence for an action of oestrogen in the preoptic area to regulate LH secretion in sheep (Blache *et al*. 1991, Scott *et al*. 1997), such co-expression is unlikely to be physiologically significant.

Puberty

One study in pigs (Ieda *et al*. 2014) found that *KISS1* mRNA levels in the arcuate nucleus did not change

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across puberty. By contrast, there is evidence that, in the sheep, kisspeptin expression increases across puberty. *KISS1* mRNA levels in the preoptic area increased across the early stages of puberty in OVX ewe lambs treated with oestrogen (Redmond *et al.* 2011*a*). In the same study, *KISS1* mRNA levels in the arcuate nucleus were not significantly different across the ages examined, but there was a significant correlation between the number of KISS1-expressing cells and the frequency of LH pulses, suggesting an association with puberty. Another study found the number of kisspeptin-ir cells was higher in post-pubertal ewe lambs (but not in ram lambs) in the arcuate nucleus (Nestor *et al.* 2012), while they found too few kisspeptin-ir cells in the preoptic area to quantify. These two studies differ in results, which might reflect both the differences in the techniques used, and the age of the animals used in the two studies (age of animals vs pre- and post-puberty). They do, however, agree in the final conclusion, that, in the ewe, changes in kisspeptin expression are associated with puberty. Further work is required to clarify the nature of this association.

Season/photoperiod

Many species of domestic animal have clear seasons of reproductive activity with polyoestrous cyclicity and sperm production coupled with periods of reproductive quiescence. An understanding of the systems that control this seasonal breeding is essential in order to manage reproduction and productivity from these species. The idea that kisspeptin neurons are regulated by season/photoperiod was first mooted by Smith *et al.* (2007), when they observed that the number of *KISS1* mRNA-containing cells was higher in the arcuate nucleus in sheep during the breeding season than during the non-breeding season. No such seasonal difference was observed in the preoptic population of kisspeptin neurons. A subsequent study (Smith *et al.* 2008*a*) found a similar difference in the number of kisspeptin-ir cells in the middle and caudal arcuate nucleus. The same result was also observed in Abadeh goat does (Jafarzadeh Shirazi *et al.* 2014) and in the mare (McGrath 2015), even though the mare is a long-day breeder, whilst sheep and goats are short-day breeders. These differences in kisspeptin cell number were matched by changes in neurokinin B cell number but not dynorphin in OVX ewes treated with oestrogen implants (Weems *et al.* 2017), indicating a seasonal change in the degree of co-localisation in KNDy neurons and presumably therefore the way they function. Notably, though, the changes in cell numbers for kisspeptin, neurokinin B and dynorphin in this study differed between ewes that were OVX and then treated with oestrogen implants and those that were OVX but without the implants. In OVX ewes, the number of kisspeptin and neurokinin B-ir cells in the arcuate nucleus was in fact lower in the breeding season than non-breeding season, (the opposite to that observed in OVX ewes treated with oestrogen (Weems *et al.* 2017). This indicates a seasonal shift in the nature of the actions of oestrogen to regulate KNDy neurons.

These seasonal differences are likely to be photoperiod driven, since transferring ewes from an artificial long-day (inhibitory) photoperiod to short days resulted in a higher number of kisspeptin neurons in the arcuate nucleus (Chalivoix *et al.* 2010), while ewes housed in short day photoperiod had higher levels of *KISS1* mRNA in the arcuate nucleus compared with ewes housed in longer photoperiods (Wagner *et al.* 2007).

Kisspeptin neurons in the ewe do not appear to express melatonin receptors (Li *et al.* 2011), suggesting that any action of melatonin to mediate the photoperiod signals to kisspeptin neurons is likely to be indirect. In sheep, dopamine neurons in the retrochiasmatic area of the hypothalamus exert an inhibitory effect on GnRH secretion during anoestrus, but not during the breeding season (Goodman *et al.* 2010). These neurons project to the arcuate nucleus (Goodman *et al.* 2010), and the kisspeptin neurons express dopamine D2 receptors (Goodman *et al.* 2012). This expression is seasonally regulated, twice as many KNDy neurons expressing this receptor in the non-breeding season (80%) as in the breeding season (40%) (Goodman *et al.* 2012). Thus, in the ewe, part of the inhibitory actions of photoperiod on kisspeptin may be via the dopamine neurons of the retrochiasmatic area.

There is now abundant evidence that thyroid hormones exert a strong influence in synchronising seasonal reproduction in a number of species, including sheep (Webster *et al.* 1991, Thrun *et al.* 1997), particularly in timing the onset of the transition from polyoestrous cyclicity to anoestrus. Part of this action may involve local conversion from thyroxine to tri-iodothyronine by type 2 deiodinases within the mediobasal hypothalamus (Yasuo *et al.* 2006). The activity of this enzyme increases with the transition to an inhibitory photoperiod in sheep (Hanon *et al.* 2008). Virtually all KNDy and preoptic kisspeptin neurons express thyroid receptor α although this expression does not change with season (Dufourny *et al.* 2015). While the expression of thyroid receptors in kisspeptin neurons may reflect the general metabolic actions of thyroid hormones, it is possible that this is

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another pathway by which season/photoperiod regulates kisspeptin neurons.

Nutrition and food intake

Nutritional status influences reproduction through alterations in GnRH/LH secretion (De Bond & Smith 2014). An understanding of this is of critical importance for animal production in order to maximise reproductive outputs such as ovulation and conception rates, and a great deal of research has been conducted in this area (Chagas *et al*. 2007, Martin *et al*. 2010). GnRH neurons receive direct inputs from neuronal systems associated with metabolic status, such as NPY, POMC (first order orexigenic and anorexigenic neurons respectively) and orexin (Norgren & Lehman 1989, Iqbal *et al*. 2001, Jansen *et al*. 2003, Goodman *et al*. 2004), allowing for direct regulation of GnRH secretion. The role of kisspeptin in relaying the influence of nutritional status on the reproductive system is unclear, and given this importance of understanding the role of nutrition in reproductive function in domestic animals it is surprising that there has not been more research conducted in this area. In one study (Backholer *et al*. 2010), lean sheep had lower *KISS1*mRNA levels in both the preoptic area and arcuate nucleus compared with control-fed ewes. This effect was reversed with intracerebroventricular (ICV) leptin infusion. This could be a direct effect of leptin on kisspeptin neurons since the same study also found the leptin receptor expressed in virtually all kisspeptin neurons in both the arcuate nucleus and preoptic area. By contrast, another sheep study (Louis *et al*. 2011) reported no phosphorylation of STAT, a marker of direct leptin receptor activation, in preoptic and arcuate kisspeptin neurons following leptin infusion, which suggests that kisspeptin neurons do not express the kisspeptin receptor. This difference may reflect the sensitivity of the techniques used. Backholer *et al*. (2010) used single cell laser microdissection, coupled with real-time PCR, which would be more sensitive than the immunohistochemistry used by Louis *et al*. (2011). Thus, it is possible that the leptin receptor is present but only expressed to a small degree. Nonetheless, there is good evidence for indirect signalling of nutritional status. Kisspeptin neurons in the arcuate nucleus of the ewe receive inputs from NPY neurons and POMC (Backholer *et al*. 2010) and ICV administration of an agonist to the POMC system increased KISS1 mRNA expression in preoptic neurons (Backholer *et al*. 2009). Similarly, high food intake increased the percentage of kisspeptin neurons receiving apposition from fibres in heifers that were immunoreactive for the POMC product,αMSH (Cardoso *et al*. 2015). Thus, the signals of nutritional status could be relayed to kisspeptin neurons via any or all of these neuronal systems.

Stress

Stressors are of great importance to domestic animal production systems, as some can have significant inhibitory actions on reproduction (Tilbrook *et al*. 2002, Einarsson *et al*. 2008), whilst some acute stress can actually stimulate reproduction, especially in pigs (Einarsson *et al*. 2008) and an understanding of this is important for animal management. Evaluation of stress effects on the reproductive system is difficult because the effects may vary with the type of stressor employed and the reproductive status of the animals (Tilbrook *et al*. 1999). Most work in this area has been conducted in sheep and pigs. There is strong evidence that some stressors, such as heat, transport isolation/restraint, barking dog, hypoglycaemia and lipopolysaccharide injection can inhibit reproduction, at least in part via an inhibition of GnRH secretion (Tilbrook *et al*. 2002, Einarsson *et al*. 2008). Part of this is through cortisol, acting via the type II glucocorticoid receptor (Ralph *et al*. 2016). GnRH neurons themselves do not express this receptor (Dufourny & Skinner 2002), but KNDy neurons do (unpublished data cited by Ralph *et al*. 2016). Further unpublished data cited by Ralph *et al*. (2016) showed that stress-like levels of cortisol increased pre-prodynorphin mRNA levels in the arcuate nucleus of ewes, compared with control animals. Lipopolysaccharide injection reduced the expression of Fos protein in kisspeptin cells in the arcuate nucleus observed at the time of the preovulatory LH surge (Fergani *et al*. 2017), indicating reduced activation of the kisspeptin neurons at this time due to the stress of the toxin. The role of stress in the regulation of preoptic kisspeptin neurons in domestic animals does not appear to have been tested.

Pheromones

In female sheep and goats, the presence of a *novel* male induces a rapid increase in the frequency of GnRH/LH pulses and thence ovulation, especially late in the anoestrous period (Hawken & Martin 2012). This phenomenon, known as the 'ram effect' in sheep, is used in some sheep production systems for flock management and accordingly important to understand fully. It is considered to be primarily an olfactory signal, although the pathway(s) to the GnRH neurons

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are poorly understood (Delgadillo *et al.* 2009). In anoestrous ewes, the ram effect resulted in an increase in *KISS1* mRNA in the rostral and mid-arcuate nucleus, but, surprisingly not the caudal arcuate (De Bond *et al.* 2013). It also resulted in an increase in the percentage of kisspeptin neurons that express Fos. Studies in OVX goats, have measured electrical activity in the region of the KNDy neurons (multi-unit activity; MUA). These have demonstrated bursts of electrical activity known as volleys and the timing of these volleys are coincident with pulses of LH secretion (Ohkura *et al.* 2009*a*, Murata *et al.* 2011). Hair from bucks (known to contain high levels of pheromones) induced an increase in MUA volleys, which were suppressed by the neurokinin B (NK3) receptor antagonist SB22200 (Sakamoto *et al.* 2013). Whilst the 'male effect' is known to also work in pigs (Kirkwood *et al.* 1981), there has been no published work considering the role of kisspeptin in its action in this species. Nonetheless, the evidence suggests that in sheep and goats, olfactory cues from novel males can exert an effect on the activity of kisspeptin neurons.

Conclusion

The use of neuroanatomical data to draw conclusions about physiological function, needs to be done with caution, as correlations do not necessarily mean causation. An increase in cell numbers and levels of mRNA expression does not necessarily mean increased cell activity, nor does the absence of changes in cell number or mRNA expression mean no change in cell activity. Nonetheless the evidence is strong that kisspeptin neurons in the arcuate nucleus are regulated by a wide range of factors from the internal and external environment in domestic animals. There is less evidence for regulation of the more rostral kisspeptin population.

Connections of Kisspeptin neurons

A number of neurons receive close contact by fibres containing kisspeptin, suggestive of synaptic input. In sheep, these include NPY and POMC (Backholer *et al.* 2010). Most attention, however, has been directed to GnRH (summarised in Table 3), where cell bodies and dendrites have received close contacts from kisspeptin fibres in sheep (Smith *et al.* 2008*a*, Lehman *et al.* 2010*b*), horses (Magee *et al.* 2009) and cattle (Tanco *et al.* 2016). There appear to be fewer inputs to GnRH cell bodies and dendrites in goats (Matsuyama *et al.* 2011). The situation in pigs is unknown.

The input to GnRH neurons was considered most likely kisspeptin itself, rather than by neurokinin B. GnRH neurons in sheep express kiss1R (Smith *et al.* 2011, Li *et al.* 2012), but they do not express the neurokinin B receptor (NK3) (Amstalden *et al.* 2010, Ahn *et al.* 2015). Surprisingly, GnRH neurons in sheep also express the kappa opioid receptor (Weems *et al.* 2016), indicating that dynorphin can act directly on GnRH neurons. It is not clear whether this reflects an action of KNDy neurons on GnRH neurons via dynorphin or the means by which non-KNDy dynorphin neurons regulate GnRH secretion. Further work is required to address this issue.

Close contacts have been observed between fibres that produce GnRH and kisspeptin in the median eminence of sheep (Lehman *et al.* 2010*a*), goats (Matsuyama *et al.* 2011), horses (Decourt *et al.* 2008) and cattle (Ohkura *et al.* 2010). These contacts were observed in the absence of the expression of synaptophysin, suggesting that there are no synaptic modifications to the membrane and hence it is likely that kisspeptin is regulating GnRH neurons in a non-synaptic manner (Matsuyama *et al.* 2011).

The number of GnRH neurons with inputs from kisspeptin fibres varies with season in sheep

Table 3 Observed inputs of kisspeptin fibres to GnRH neurons & fibres in domestic species.

	Species	Reference
GnRH cells		
	Sheep	Smith *et al.* (2008*a*), Lehman *et al.* (2010*b*), Li *et al.* (2012), Merkley *et al.* (2015)
	Goat	Matsuyama *et al.* (2011)
	Horse	Decourt *et al.* (2008), Magee *et al.* (2009)
	Cattle	Tanco *et al.* (2016)
	Pig	Not tested
GnRH fibres in median eminence		
	Sheep	Lehman *et al.* (2010*b*), Smith *et al.* (2011), Merkley *et al.* (2015)
	Goat	Matsuyama *et al.* (2011)
	Horse	Decourt *et al.* (2008)
	Cattle	Ohkura *et al.* (2010)
	Pig	Not tested

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(Smith *et al.* 2008*a*), with a greater percentage of GnRH-ir cells in the mediobasal hypothalamus receiving kisspeptin input in the breeding season than the non-breeding season in OVX ewes treated with oestrogen. By contrast, we (McGrath 2015) found no season difference in the proportion of GnRH neurons that were apposed by kisspeptin fibres in the mare, comparing non-breeding season, vernal transition and breeding season.

An alternative means by which kisspeptin could influence the reproductive system is at the anterior pituitary gland to influence gonadotrophin secretion. Kisspeptin has been measured in hypophyseal portal blood from OVX ewes, albeit at low levels (Smith *et al.* 2008*b*). Furthermore, gonadotrophs from ewes express the kiss1 receptor (Smith *et al.* 2008*b*). Thus, while the only available information for domestic animals is from sheep, the evidence is that kisspeptin can make contact with, and influence gonadotroph function.

In summary, kisspeptin neurons are well placed to influence gonadotrophin secretion, as they make contact with GnRH neurons at the cell bodies, dendrites and terminals, as well as, in an endocrine manner, directly with gonadotrophs. Thus, they are in a position to influence GnRH and/or LH secretion. Whether they do so is discussed in the next section.

Kisspeptin regulation of the hypothalamo–pituitary–gonadal axis

Most research into kisspeptin in domestic animals has centred on its ability to influence LH secretion. Almost all of this research has been conducted in females, with very little research centred on the role of kisspeptin in the regulation of LH secretion in males. The quantity of research in this area is substantial, and it is not possible for an exhaustive review of all relevant studies. A number of excellent reviews have more than adequately covered this material, including (Caraty *et al.* 2010, Caraty *et al.* 2012, Okamura *et al.* 2013).

The kisspeptin peptide can be detected in several forms, from 10-54 amino acids in length, but the biological activity appears to reside in the last 10 amino acids of the C terminal (Gottsch *et al.* 2004). It is this 10 amino acid fragment that is used for almost all studies on the actions of kisspeptin. Single intravenous (IV) bolus treatment of kisspeptin has increased plasma LH in females from all the species under consideration – sheep (Caraty *et al.* 2007), goats (Hashizume *et al.* 2010), horses (Magee *et al.* 2009), cattle (Kadokawa *et al.* 2008) and pigs (Lents *et al.* 2008). Longer IV infusions have also stimulated LH secretion in ewes (Caraty *et al.* 2007) and mares (Decourt *et al.* 2014, McGrath *et al.* 2016). IV kisspeptin also increased plasma LH in male goats (Saito *et al.* 2012) and bull calves (Ezzat *et al.* 2009), as well as testosterone in stallions (Akhtar *et al.* 2017). Surprisingly, little other research has been conducted on the actions of kisspeptin in males of domestic animals.

The action of kisspeptin to stimulate LH secretion is likely to be at one of three places; stimulating GnRH secretion through actions at the GnRH neurons in the preoptic area or arcuate nucleus (cell bodies, dendrites or dendrons), on GnRH terminals in the median eminence, or stimulating LH secretion directly in the pituitary gland. This can be to influence the generation of GnRH/LH pulses as well as the preovulatory GnRH/LH surge. The evidence for each is discussed in turn.

Kisspeptin action at the pituitary gland

Studies using primary pituitary cell cultures from ewes (Smith *et al.* 2008*b*), male calves (Suzuki *et al.* 2008) and barrows (Suzuki *et al.* 2008) have demonstrated that kisspeptin can directly stimulate LH secretion from pituitary cells. On the other hand, kisspeptin was unable to influence the LH response to GnRH in ewes that had undergone hypothalamo–pituitary disconnection, to isolate the pituitary gland from the influence of the hypothalamus (Smith *et al.* 2008*b*). This gives reason to question whether the capability of kisspeptin to act at the pituitary gland is of physiological importance, at least in the ewe.

Kisspeptin action on GnRH secretion

There is strong evidence that kisspeptin regulates GnRH secretion. IV kisspeptin injection increased GnRH secretion in hypophyseal portal blood in ewes (Smith *et al.* 2011, Caraty *et al.* 2013) and OVX goats (Tanaka *et al.* 2012). Similarly, IV (Caraty *et al.* 2007) or ICV (Messager *et al.* 2005) kisspeptin treatment in ewes produced an increase in GnRH secretion in cerebrospinal fluid (CSF), with a parallel rise in plasma LH concentrations. Thus, kisspeptin stimulates GnRH secretion, at least in sheep and goats. The question is whether this is an action at or near the GnRH cell bodies or the terminals. The anatomical evidence is that either or both are possible (*vide supra*). A large IV bolus injection of kisspeptin in ewes increased kisspeptin levels in peripheral blood but not in CSF (Caraty *et al.* 2013). This indicates that IV kisspeptin does

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not cross the blood brain barrier. As such, it is unlikely to act on or near GnRH cell bodies or dendrites, although it is still able to act on GnRH terminals in the median eminence. Indeed, there is good evidence for kisspeptin action in the median eminence. Kisspeptin stimulated GnRH release from cultured median eminence explants from OVX ewes (Smith *et al*. 2011), and microinjection of kisspeptin into the median eminence *in vivo* stimulated a pulse of LH secretion in OVX ewes (Ezzat *et al*. 2015). In addition, in a study using anaesthetised animals, which precludes a central action, IV kisspeptin treatment of OVX ewes still increased LH secretion (Ezzat *et al*. 2015).

Whilst IV kisspeptin treatment is unlikely to act on GnRH cell bodies or dendrites to stimulate GnRH secretion, this does not prevent endogenous kisspeptin from acting in this way, and ICV kisspeptin, which also stimulates LH secretion in sheep (Messager *et al*. 2005) and pigs (Lents *et al*. 2008), could act at this site. The anatomical evidence for contact between kisspeptin fibres and GnRH cell bodies and the presence of kiss1R on GnRH cells (*vide supra*) indicates that an action of kisspeptin on GnRH cell bodies/dendrites would seem likely. The best evidence from domestic animals probably comes from studies using the 'ram effect' (De Bond *et al*. 2013). Pulsatile GnRH/LH secretion in anoestrous ewes rapidly increases following exposure to a novel ram. Such an exposure induced Fos protein in both kisspeptin and GnRH neurons, indicating that both kisspeptin and GnRH neurons were activated by this event. This would not have happened if it were purely an action on GnRH terminals.

GnRH pulse generator

GnRH secretion is pulsatile in nature (Clarke & Cummins 1982) and a great deal of research has been undertaken to determine the source of this pulsatile activity; the so-called GnRH pulse generator. There is evidence that the network of KNDy neurons contributes to GnRH pulse generation and might even be the GnRH pulse generator. Early evidence for this hypothesis is described by Lehman *et al*. (2010*a*) and proposes that synchronous activity of KNDy neurons involves the coordinated activity of neurokinin B and dynorphin, with the output signal to the GnRH neurons being kisspeptin. The signal to stimulate a GnRH pulse is initiated by neurokinin B activity within the KNDy neuron network, while dynorphin acts to cease kisspeptin release from the KNDy neurons, which acts to end GnRH pulses. The anatomical evidence described above, whereby KNDy neurons express both the neurokinin B receptor (NK3R) and the dynorphin receptor (Kappa opioid receptor) but not the kiss1R, supports this hypothesis, as does evidence that Kappa opioid receptors are internalised at the time of GnRH/LH pulses (Weems *et al*. 2018).

In vivo electrophysiological studies conducted in goats provide a good model in which to test these hypotheses. Electrodes implanted into the mediobasal hypothalamus, the home to the KNDy neurons, consistently record volleys in electrical activity (MUA), as described earlier in this paper. They are considered to reflect the activity of the KNDy neuronal network although these cannot be directly determined. Nonetheless, these increases in activity consistently coincide with pulses in LH secretion in OVX goats (Ohkura *et al*. 2009*b*, Wakabayashi *et al*. 2010, Sakamoto *et al*. 2013), are identical in size and shape on both sides of the brain and have been termed GnRH pulse generator activity. IV kisspeptin treatment to OVX goats increased LH pulses with no change to MUA, suggesting that the source of the MUA is upstream of kisspeptin action (Ohkura *et al*. 2009*b*). Central (ICV) infusion of dynorphin decreased MUA volleys (and LH pulses), while neurokinin B induced volleys in MUA, suggesting that neurokinin B and dynorphin are involved in generating a rhythmic discharge of kisspeptin (Wakabayashi *et al*. 2010). Conversely, central infusion of a neurokinin B antagonist blocked pheromone-induced increases in MUA volleys, whilst blocking pulsatile LH secretion, suggesting that communication between the KNDy cells is needed for MUA volleys and LH secretion (Sakamoto *et al*. 2013). Similarly, following suppression of LH secretion with kisspeptin receptor disruption in OVX goats, male pheromones or treatment with the neurokinin B agonist senktide, which normally stimulate LH pulses, induced MUA volleys but no increase in LH pulses (Yamamura *et al*. 2014). This suggests actions upstream of the final kisspeptin output. In support of these results are studies conducted in ewes (Goodman *et al*. 2013, Porter *et al*. 2014, Li *et al*. 2015), where kiss1R or neurokinin B receptor (NK3R) antagonists blocked LH pulses, suggesting a stimulatory role for those receptors, whilst neurokinin B itself, other NK3R agonists or a dynorphin antagonists stimulated LH pulses.

An alternate hypothesis, which is not mutually exclusive of the hypothesis of the KNDy neuronal network as the GnRH pulse generator, is that there is inherent pulsatility in GnRH neurons themselves, which just needs tuning. Evidence for this in sheep comes from a study where LH pulsatility was blocked in OVX ewes by treatment with an NK3R antagonist, and then restored with an IV infusion of kisspeptin (Clarke *et al*. 2018).

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In that study, the KNDy neuronal network was effectively shut down, yet constant delivery of kisspeptin reactivated LH pulses. This argues that there is pulse generation capacity upstream of the kisspeptin receptor. It is possible that there is GnRH pulse generation capacity in both the KNDy neurons and GnRH neurons and that they can work together or independently, but more work is required to understand the true nature of the so-called GnRH pulse generator.

Kisspeptin regulation of the LH surge and ovulation

The role of kisspeptin in the regulation of preovulatory surge of LH appears to vary with species, which may relate to the varying nature of the LH surge. In sheep, a preovulatory-like LH surge can be easily and reliably produced in anoestrous or OVX ewes with a single injection of oestradiol benzoate (Wright & Clarke 1988), with an LH surge commencing 12–15 h later. Preovulatory LH surges can likewise be generated in progesterone pre-treated ewes with IV infusions of kisspeptin for 30–48 h in anoestrous ewes, and in even less time in ewes during the breeding season (Caraty *et al*. 2007, Sébert *et al*. 2010). This is most likely not the direct generation of an LH surge, but an increase in GnRH/LH pulses, which stimulates the ovary, leading to an LH surge stimulated by endogenous oestrogen (Sébert *et al*. 2010). Similarly, hourly kisspeptin injections for 24 h in lambs triggered an LH surge and ovulation (Redmond *et al*. 2011*b*). Meanwhile, ICV treatment with kisspeptin antagonist p-271 attenuated the size of an oestrogen-induced LH surge (Smith *et al*. 2011). Collectively, these results strongly indicate a role for kisspeptin in the generation of the LH surge in sheep, but this latter result, where the LH surge was attenuated, but not blocked completely, indicates that kisspeptin is not solely responsible for the generation of the LH surge.

The LH surge in horses is more complex, developing slowly over several days (Geschwind *et al*. 1975) and ovulation is hard to induce clinically (Norman & Larsen 2010). It is perhaps therefore not surprising that providing evidence for a role of kisspeptin in the LH surge has proven more challenging. A single IV injection of a large dose of kisspeptin (10 mg) did advance ovulation in mares with large ovarian follicles (Briant *et al*. 2006). Lower doses of kisspeptin, however, whether as bolus injections or continuous infusions, up to 9 days in length and across a range of doses, all increased circulating LH concentrations, but failed to induce an LH surge (Magee *et al*. 2009, Decourt *et al*. 2014, McGrath *et al*. 2016), whether in cycling mares, anoestrous mares or those in vernal transition. Thus, the role that kisspeptin plays in the generation of the LH surge in horses is not clear. Considering that experiments using the same model that consistently induced an LH surge in sheep failed to induce one in horses, this indicates that the role of kisspeptin in horses differs from that in sheep.

Conclusion

A model that integrates this data is illustrated in Fig. 1. Whilst there are both species and sex differences,

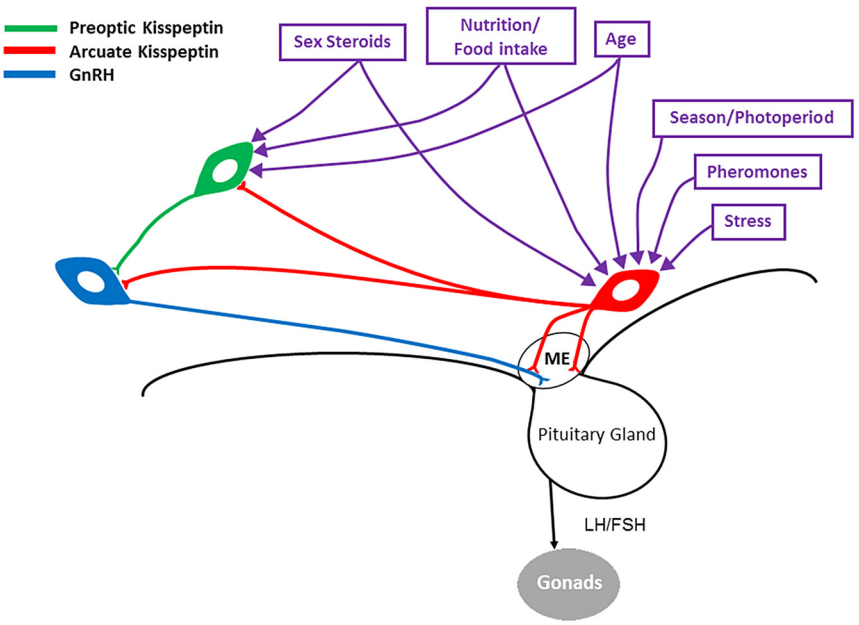

Figure 1
A model for the integration of factors from the internal and external environment in the brain control of reproduction in domestic animals. These factors are integrated by KNDy neurons in the arcuate nucleus, and the kisspeptin neurons in the rostral hypothalamus. These neurons then input to the GnRH neurons at the terminals in the median eminence and also at the cell bodies/dendrites to regulate GnRH secretion into the hypophyseal portal vasculature. In addition, kisspeptin is released in to the portal vasculature itself, where it may directly regulate gonadotroph secretion.

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the evidence is very strong that kisspeptin exerts a role in the regulation of GnRH and LH secretion in domestic animals, most likely influencing both the generation of GnRH pulses, as well as the preovulatory LH surge. This action occurs at the median eminence to influence the release of GnRH into the hypophyseal portal vasculature. In addition, it may also act on GnRH cell bodies and dendrites, as well as at the anterior pituitary gland although the evidence is less strong. At least part of this action is a direct one, with kisspeptin fibres making contact with GnRH neurons. In doing so, the kisspeptin neurons integrate a lot of information about the internal and external environment of the animals. As such, it is possible that the kisspeptin neuronal network is the true controller of the reproductive system, with the GnRH neurons being the primary output signal from the brain.

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