The role of kisspeptin and gonadotropin inhibitory hormone (GnIH) in the seasonality of reproduction in sheep

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Sheep are seasonal breeders and reproductive status is controlled by photoperiod. Recent recognition of the significant role for kisspeptin and gonadotropin inhibitory hormone (GnIH) in the regulation of gonadotropin releasing hormone (GnRH) cells has provided a new perspective in the seasonal regulation of reproductive activity. Virtually all kisspeptin cells express estrogen receptors and kisspeptin is a potent stimulator of GnRH secretion. Thus, kisspeptin cells provide a conduit by which changes in estrogen feedback effects may be exerted upon GnRH cells. Changes in the activity of kisspeptin cells with season indicate a major role in the seasonal changes in reproductive activity in the ewe. GnIH is an inhibitor of reproductive function and there is mounting evidence that changing activity of this system is also an important determinant of reproductive status. Reciprocal changes in kisspeptin and GnIH activity explain seasonal changes in the function of GnRH cells.

Introduction

Sheep display seasonal patterns of reproduction which is regulated by day length (Robinson 1959). A component of the mechanism that causes a seasonal change in the frequency of luteinizing hormone (LH) pulses was shown in ovariectomised (OVX) ewes, thus being independent of any action of gonadal steroids (Robinson et al. 1985). Earlier work showed that there is an estrogen dependent mechanism that also underlies the transition between breeding and non-breeding seasons (Legan et al. 1977). With development of a model in sheep to measure secretion of gonadotropin releasing hormone (GnRH), it was then possible to attribute the pulses in peripheral plasma levels of LH to pulses of GnRH from the hypothalamus (Clarke and Cummins 1982) Measurements of hypophysial portal plasma concentrations showed that estrogen exerted an enhanced negative feedback effect during the non-breeding season (Karsch et al. 1993).

Gonadal steroid feedback on GnRH secretion governs the reproductive axis (Karsch et al. 1987, Clarke 1993). One of the most fundamental aspects of the operation of the hypothalamo-pituitary gonadal (HPG) axis is the means by which sex steroids act to modulate GnRH secretion and this took some time to resolve. Because GnRH cells do not possess the relevant sex steroid receptors (Herbison 1998), significant efforts were made in various laboratories and species over 3 decades (1970's to 2003) to identify steroid-receptive elements in the brain that relayed feedback information to the GnRH cells. Various cell types were found to express estrogen, pro-

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gesterone and androgen receptors, but evidence of a major conduit remained elusive (Tilbrook et al. 2002). The discovery that kisspeptin and its cognate receptor are essential for normal reproduction (de Roux et al. 2003, Seminara et al. 2006) changed this, as discussed below. These cells provide stimulatory input to the GnRH cells, allowing transmission of sex steroid feedback regulation of reproductive function. Other cells may also participate in sex steroid feedback as well as modulation of GnRH cells by season, stress, immune status, nutritional status etc. Thus, a number of neuronal systems converge on the GnRH cells to determine the output of these cells in terms of GnRH secretion.

Another major advance in our understanding of the secretion and action of GnRH, albeit less well recognized, was the discovery of gonadotrophin inhibitory hormone (GnIH) in the hypothalamus of the quail (Tsutsui et al. 2000). This challenged the concept that gonadotropin secretion was controlled by a singular hypothalamic factor. Evidence that GnIH is important in mammals is now irrefutable. Like kisspeptin, GnIH is an RF-amide peptide but it exerts negative effects on GnRH cells (Ducret et al. 2009) and, at least in some species, the gonadotropes (Clarke et al. 2008). This review will summarise the general properties of these peptides and then evaluate their roles in the regulation of seasonal breeding with special reference to the sheep as a model.

General consideration of the control of GnRH Cells

GnRH neurons exhibit repetitive bursts of action potentials, consistent with phasic secretory activity (Suter et al. 2000). This is translated into the secretory mode as demonstrated by the phasic release of GnRH from cells of the fetal nasal placode of rhesus monkeys studied in culture (Terasawa et al. 1999). In addition, there is communication between GnRH neurons allowing for co-ordinate control (Campbell et al. 2009). If this phasic property of the cells is converted into bursts of secretion of GnRH, then it must be regulated in some fashion, to explain the differences in the patterns of secretion that are seen, for example, during the estrous cycle. The primary means of control is via feedback effects of gonadal steroids.

GnRH cells receive input from brain stem noradrenergic cells (Pompolo et al. 2003a, Rawson et al. 2001), serotoninergic elements of the raphe nucleus (Kiss and Halasz 1985) and a variety of cells of the hypothalamus and the forebrain (Iqbal et al. 2001, Pompolo et al. 2005, Pompolo et al. 2003a, Pompolo et al. 2003b). In addition to direct input to GnRH cells, control may be exerted by systems that form inter-neuronal pathways. Thus, the influence of the estrogen receptive A1 noradrenergic neurons of the brainstem may involve multisynaptic relay via the bed nucleus of the stria terminalis (BNST) or preoptic regions in close vicinity to the GnRH cells (Pereira et al. 2010, Pompolo et al. 2005). Anterograde and retrograde neuronal tracing between the arcuate nucleus of the hypothalamus (ARC) and the preoptic area of the ovine brain indicate that there is very limited direct input to GnRH cells from the former (Backholer et al. 2010, Pompolo et al. 2001). In this regard, even though there is very strong evidence that cells of the ARC play a significant role in the regulation of GnRH cells, there is little evidence of direct neuronal projections that subserve this (Backholer et al. 2010). One caveat on this observation is that ARC cells may project to dendrites of GnRH cells that are not readily seen with standard histolological techniques. The existence of inter-neuronal pathways to GnRH neurons from various regions of the brain would allow complex multifactorial control of the reproductive system, incorporating information in relation to metabolic status, season, stress, immune status, olfactory stimuli etc.

Noradrenergic cells of the brain stem, and forebrain glutamatergic cells and inhibitory cells utilising gamma amino butyric acid (GABA) as a transmitter are important in the regulation

of GnRH secretion (Constantin et al. 2010, Kuehl-Kovarik et al. 2002, Pompolo et al. 2003a, Herbison 1997), but these cells do not fulfil all criteria required for mediation of feedback regulators; space does not allow full dissertation on this issue. In 2003, the revelation that kisspeptin cells were essential for reproductive function led to a significant revision in our understanding of how the function of GnRH cells is controlled by 'upstream' elements. This was followed by the gradual acceptance of the role of GnIH, which had been identified 3 years earlier.

Kisspeptin and the control of GnRH cells

There are two major groupings of kisspeptin cells in the mammalian brain, one being in the ARC and the other being in the rostral hypothalamus/POA region. In rodents, the latter group of cells is located in the anteroventral periventricular nucleus (AVPV) and preoptic periventricular nucleus (PeV) (Clarkson et al. 2008, Gottsch et al. 2004, Smith et al. 2005a, Smith et al. 2005b). In the ovine brain, the two synonymous populations of kisspeptin cells are found in the ARC and in the dorsolateral POA (Franceschini et al. 2006). In the ovine brain, kisspeptin cells of the POA provide direct input to GnRH neurons, whereas kisspeptin cells of the ARC may regulate GnRH neurons through an inter-neuronal pathway involving cells of another type (Backholer et al. 2010).

Amongst the neuronal elements that *modulate* GnRH neurons, kisspeptin achieved prominence for a number of reasons. Inactivating mutations in either the gene for kisspeptin (*Kiss1*) or the cognate receptor (GPR54) cause loss of reproductive function (de Roux et al. 2003, Seminara et al. 2003). Supporting the notion that kisspeptin exerts direct action on the GnRH cells, virtually all of these cells express the cognate receptor (GPR54) (Han et al. 2005, Irwig et al. 2004). Indeed, GPR54 is expressed in virtually all GnRH cells in the ewe brain (Smith et al. 2009). Blockade of these receptors with a kisspeptin antagonist, prevents pulsatile LH secretion in a number of species (Roseweir et al. 2009). Other work shows that kisspeptin directly stimulates GnRH secretion (Messager et al. 2005).

In sheep, kisspeptin does not appear to affect LH or FSH release by direct action on pituitary gonadotropes (Smith et al. 2008b). This is in spite of the fact that at least some GPR54 expression can be detected in the pituitary gland by PCR (Smith et al. 2008b). Consistent with this, kisspeptin is not secreted into the hypophysial portal blood in significant amounts (Smith et al. 2008b). Interestingly, however, kisspeptin cells project to the median eminence, where varicose fibres come into close apposition to GnRH fibres (Ramaswamy et al. 2008) and it is possible that there is axo-axonic regulation of GnRH secretion at this level (Keen et al. 2008). Evidence for the action of kisspeptin at the level of the median eminence has been demonstrated in the non-human primate (Keen et al. 2008), based on concordance of pulses of GnRH and kisspeptin in push-pull samples as well as the demonstration that kisspeptin injection to the median eminence stimulated LH secretion. Regarding the latter finding however, it is equivocal as to whether kisspeptin acted on GnRH cell bodies in the mediobasal hypothalamus or whether it acted on secretory terminals.

Kisspeptin cells transmit the negative feedback effect of sex steroids to GnRH cells

GnRH cells do not express estrogen receptor- α (ERα), or androgen receptors (Herbison et al. 1996, Huang and Harlan 1993), but it is clear that sex steroids regulate the secretion of GnRH. In the ovine ARC, virtually all kisspeptin cells express estrogen and progesterone receptors, but only 50% of the kisspeptin population of cells in the lateral POA express ERα (Franceschini

et al. 2006). The question arises as to what function is performed by the kisspeptin cells that do not express steroid receptors. As mentioned above, there is good evidence that cells of the ovine ARC do not project directly to GnRH cells, but may exert influence on the latter by an inter-neuronal pathway. On the other hand, kisspeptin cells in the lateral POA of the ovine brain do appear to provide direct neuronal input to GnRH cells (Backholer et al. 2010).

Following ovariectomy, up-regulation of *Kiss1* expression is seen in the ARC kisspeptin cells (Smith et al. 2007) and increased numbers of kisspeptin cells are seen by immunohistochemistry (Pompolo et al. 2006). This shows that chronic effects of gonadal steroids restrain the ARC cells, but not the POA cells, and the effect of ovariectomy is reversed by chronic estrogen treatment (Smith et al. 2009). Virtually all kisspeptin cells in the ARC co-express dynorphin (DYN) and neurokinin B (NKB) (Goodman et al. 2007). This has led to the naming of these cells as K (kisspeptin) N (neurokinin B) Dy (dynorphin) (KNDy) cells (Cheng et al. 2010). The KNDy cells also express both ERα and progesterone receptor at a high level (Franceschini et al. 2006, Foradori et al. 2002), providing the necessary machinery for KNDy cells to mediate both negative and positive feedback effects of sex steroids. There is good evidence that dynorphin plays a role in mediating the negative feedback effect of progesterone (Foradori et al. 2005, Goodman et al. 2004), in addition to the evidence that chronic estrogen treatment down-regulates KNDy cells in OVX ewes (Smith et al. 2009). Further support for the notion that these cells participate in transmitting the negative feedback effect to GnRH cells is the observation that kisspeptin expression in the ARC is reduced in the luteal phase of the estrous cycle (Smith et al. 2009). This does not mean that other cells, such as those that produce enkephalin, may also participate in the negative feedback regulation of GnRH secretion (Walsh et al. 2001), but a major role for the KNDy cells is most likely. As for the involvement of these cells in the negative and positive feedback mechanism, any transmission from the ARC to the GnRH cells of the POA is likely to involve an inter-neuronal pathway. KNDy cells may regulate a subset of GnRH cells in the mediobasal hypothalamus, to exert the negative feedback effect (Goodman et al. 2004).

Kisspeptin and seasonal reproduction

Expression of *Kiss1* in the ARC of ovary-intact ewes is elevated at the onset of the breeding season, as evidenced by manipulations in photoperiod (Wagner et al. 2008). Here, *Kiss1* expression was 3 times higher on a photoperiod of 8 light and 16 dark (8L:16D) than that in animals on longer photoperiods. It is possible this effect could represent the effects of seasonal endogenous steroid production. Importantly, further studies in ewes show even in the absence of gonadal feedback (in OVX ewes with or without chronic estrogen implants) *Kiss1* mRNA and the number of kisspeptin immunoreactive cells is greater in the ARC during the breeding season than in the anestrous season (Smith et al. 2007, Smith et al. 2008a). Moreover, the inhibitory effects of chronic estrogen treatment on *Kiss1* mRNA and kisspeptin expression in the ARC (indicative of negative feedback) are greater during the non-breeding season (Smith et al. 2008a). These data suggest the seasonal change in sensitivity to estrogen, which is a major mechanism for seasonal reproduction, is effected, at least in part, by changing responsiveness of the kisspeptin cells to estrogen. Interestingly, the seasonal effect on *Kiss1* and kisspeptin expression was seen in the ARC, but not the POA. Alternatively, a small, yet significant effect of photoperiod was seen in kisspeptin cells in the POA of ewes transferred to short-day photoperiod (replicating the shift to the breeding season)(Chalivoix et al. 2010). Not surprisingly, a clear increase in kisspeptin cellular expression was also seen in the ARC. The authors suggest that the increase in kisspeptin expression stems from increased kisspeptin synthesis (Chalivoix et al. 2010).

In addition to the seasonal change in *Kiss1* expression and kisspeptin synthesis, the extent to which kisspeptin cells provide input to the GnRH neurons is greater during the breeding season than in the non-breeding season (Smith et al. 2008a). Presumably this input arises from the POA kisspeptin cells although this has not been tested by determining whether the increased input is due to 'recruitment' of cells in the arcuate nucleus. Thus, both the level of kisspeptin expression and the level of kisspeptin input to GnRH neurons are higher during the breeding season, while the negative feedback effects of estrogen on kisspeptin are lower at this time of the year. This is a strong indication that kisspeptin cells play a fundamental role in the seasonal regulation of reproduction.

As the seasonal change in kisspeptin expression in ewes is replicated by manipulation of photoperiod, it appears this may be the primary stimulus governing kisspeptin change. Work in seasonal rodents also provides strong evidence that the seasonal change in kisspeptin levels is due to alterations in photoperiod (Revel et al. 2006, Simonneaux et al. 2008). More importantly, these rodent studies further indicate the photoperiod change in kisspeptin is driven by changes in the pattern of melatonin secretion. Whether this is true in the ovine is not yet determined. Whether melatonin is able to exert its effects directly on kisspeptin cells is not yet known as there are no data to indicate kisspeptin cells possess melatonin receptors.

Role of the A14/A15 dopaminergic nucleus

Dopaminergic neurons located in the A15 region of the hypothalamus are thought to provide considerable input toward the increase in estrogen negative feedback sensitivity during the non-breeding season (for review see (Goodman et al. 2010). These cells are known to hold LH pulse frequency in check during the non-breeding season, but not the breeding season (Havern et al. 1994, Meyer and Goodman 1985). Furthermore, A15 dopaminergic cells are activated by estrogen only during the non-breeding season, but they do not express ERα (Lehman et al. 1996, Lehman and Karsch 1993). Estrogen responsive afferents to these cells may include glutamatergic cells of the POA (Singh et al. 2009).

In spite of the evidence that A14/A15 dopaminergic cells are key players in the seasonal changes in fertility in sheep, these cells do not appear to project directly to GnRH cell bodies (Tillet et al. 1989). Anterograde tracing experiments reveal A15 dopaminergic cells project predominantly to the caudal ARC and the median eminence (Goodman et al. 2010). It is enticing to speculate that the A15 dopaminergic cell projections to the former, communicate directly with kisspeptin cells located in this region, alter their expression and activity, and thereby govern seasonal reproduction.

Gonadotropin inhibitory hormone (GnIH)

GnIH was identified as a hypothalamic factor that inhibits the HPG axis in the quail (Tsutsui et al. 2000) and the role of this peptide is well established for avian species (Tsutsui 2009). It is now clear GnIH also plays an important role in the regulation of reproduction in mammals (Clarke et al. 2009, Smith and Clarke 2010). Mammalian GnIH was first named RF-amide related peptide (RFRP), being a member of the RF-amide family (Hinuma et al. 2000), but it is suggested the original nomenclature (GnIH) should apply to all species (Clarke et al. 2009, Smith and Clarke 2010).

In the sheep brain, *in situ* hybridization identified GnIH-expressing cells in the ventral region of the paraventricular nucleus and the dorsomedial nucleus (Clarke et al. 2008, Dardente et al. 2008, Smith et al. 2008a), with a similar distribution detected using immunohistochemistry,

using either a white crowned sparrow GnIH antiserum (Kriegsfeld et al. 2006, Smith et al. 2008a) or an antiserum raised in guinea pigs against human GnIH-3 (Qi et al. 2009). Between 40-80% of GnRH cells show GnIH-immunoreactive varicose fibers in close proximity in the primate, rat, sheep, hamster and mouse brain (Johnson et al. 2007, Kriegsfeld et al. 2006, Qi et al. 2009, Smith et al. 2008a, Wu et al. 2009, Ubuka et al. 2009). This provides a substrate by which GnRH cells may be regulated either directly or indirectly by GnIH. Indeed, GnIH has been shown to directly inhibit the electrical properties of GnRH cells in mice (Wu et al. 2009, Ducret et al. 2009). GnIH-immunoreactive terminals have also been observed in the neurosecretory zone of the median eminence in hamsters, sheep, and primates (Clarke et al. 2008, Dardente et al. 2008, Kriegsfeld et al. 2006, Ubuka et al. 2009). On this basis, a hypophysiotropic role for GnIH is proposed for the sheep (Clarke et al. 2008).

GnIH action on the pituitary gonadotropes

Intravenously (iv) administered GnIH-3 had no effect on basal LH secretion in OVX rats and only minimal (albeit statistically significant) effects on GnRH-stimulated secretion (Rizwan et al. 2009); this was interpreted to mean that GnIH has no major effect on gonadotropes. Others (Murakami et al. 2008) showed a reduction in plasma LH levels in OVX rats 2 h after iv administration, with lack of effect of icv administration. In OVX ewes, systemically administered GnIH-3 reduced pulsatile LH secretion (Clarke et al. 2008), without any effect on the plasma levels of other pituitary hormones, such as growth hormone or prolactin; similar results have also been obtained in the bovine (Kadokawa et al. 2008).

The dose-dependent reduction in GnRH-stimulated LH secretion that is seen in cultures of rat, sheep and bovine pituitary cells, supports the notion of a direct effect of GnIH on gonadotropes (Clarke et al. 2008, Kadokawa et al. 2008, Murakami et al. 2008), although some contrary data have also been reported in studies on rat pituitary cells *in vitro* (Anderson et al. 2009). Such dissimilar results may be due to variable culture conditions or may relate to possible species differences in peripheral GnIH activity. Other lines of evidence also suggest direct pituitary action of GnIH in sheep. Firstly, GnIH-3 eliminates the GnRH-stimulated mobilisation of intracellular calcium in gonadotropes, which is considered mandatory for gonadotropin release (Clarke et al. 2008). Second, GnRH-stimulated up-regulation of *LHβ* mRNA levels is also negated by GnIH, which may be due to reduced phosphorylation of extracellular signal-regulated kinase (ERK) (Sari et al. 2009). These data indicate a direct action of GnIH on the pituitary gonadotrope to reduce both synthesis and secretion of LH. *In vitro* treatment of ovine pituitary cell cultures also show an effect of GnIH-3 to reduce FSH secretion in response to GnRH (Clarke et al. 2008) as well as reducing *FSHß* mRNA levels (Sari et al. 2009). Thus, GnIH may inhibit the production/secretion of both gonadotropins.

GnIH and seasonal reproduction

In ewes, a lower number of immunoreactive GnIH cells is detected during the breeding season than in the non-breeding season, but no change was seen in cellular GnIH mRNA expression assessed by *in situ* hybridization (Smith et al. 2008a). Contrary to this, GnIH mRNA expression in Soay ewes (also assessed by *in situ* hybridization) was greater in animals held at a long-day (16L:8D) photoperiod than in those on short day (8L:16D) photoperiod (Dardente et al. 2008). Nevertheless, when these ewes were held on extreme long day photoperiods (22 h light), the effect on GnIH gene expression was lost. It was concluded that GnIH may not play

a major role in seasonality (Dardente et al. 2008), but the inhibitory effect may be amplified by an increase in input to GnRH cells during the non-breeding season (see below)(Smith et al. 2008a). Moreover, after transferring ewes to a long-day photoperiod, no effect on GnIH gene expression was apparent in time-points examined before 42 days (Dardente et al. 2008). This lack of a short-term effect is consistent with the notion that the transition between the breeding and non-breeding seasons requires approximately 60 days (Lincoln 1999), and also indicates that the full seasonal transition (and change in GnIH expression) in these ewes may not have occurred.

The lower level of GnIH producing cells that is seen during the breeding season is accompanied by a reduction in the number of GnRH cells contacted by GnIH terminals (Smith et al. 2008a). Given the role of GnIH on the reproductive system, the net effect of these data would indicate the activity of GnIH may be a contributing factor to the inhibition of the reproductive system during the non-breeding season. Further work, including measurement of GnIHR expression in different seasons may be informative.

Kisspeptin and GnIH as key reciprocal regulators in seasonal breeding

Sheep are seasonal breeders, being sexually active in response to short day photoperiod (Karsch et al. 1984, Malpaux et al. 1998). Whereas it has been known for some years that seasonality in sheep is due to alterations in the frequency of generation of GnRH/LH pulses (Robinson et al. 1985), as well as an alteration in the negative feedback effect of ovarian steroids (Legan et al. 1977), there was no identification of a neural substrate that changes with season and controls GnRH secretion accordingly. The A15 dopaminergic nucleus was identified as a key centre in the control of seasonality (Thiery et al. 1995) but as to how this is connected to GnRH secretion is not yet clear. With the revelation of key regulatory function of kisspeptin and GnIH, it is hardly surprising that this has been investigated in relation to seasonal breeding. In the ewe, kisspeptin production (ARC) and input to GnRH cells is reduced in seasonal anestrus and increases at onset of the breeding season (Smith et al. 2008a). Also in the ewe, GnIH protein expression is higher during the non-breeding season than in the breeding season as determined by the number of cells stained with immunohistochemistry (Smith et al. 2008a). Terminal projections from GnIH cells to GnRH neurons are increased during the non-breeding season (Smith et al. 2008a).

Conclusion

Reciprocal changes in kisspeptin and GnIH activity indicate that both RF-amide peptides play a role in seasonal changes in reproductive activity. Kisspeptin treatment of anestrous ewes causes ovulation, offering a potential means by which seasonal acyclicity can be overcome (Caraty et al. 2007). Intervention studies such as reducing GnIH function in the non-breeding season would be instructive.

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