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Neural Systems Mediating Seasonal Breeding in the Ewe

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Abstract

Seasonal reproduction in ewes is caused by a dramatic increase in response to oestradiol (E₂) negative feedback during the non-breeding (anoestrous) season. Considerable evidence supports the hypothesis that A15 dopaminergic (DA) neurones in the retrochiasmatic area (RCh) play a key role in these seasonal changes. These A15 neurones are stimulated by E₂ and inhibit GnRH secretion in anoestrus, but not the breeding season. Because A15 neurones do not contain estrogen receptors- α (ER α), it is likely that E₂-responsive afferents stimulate their activity when circulating E₂ levels increase during anoestrus. Retrograde tract tracing studies identified a limited set of ER α -containing afferents primarily found in four areas (ventromedial preoptic area, RCh, ventromedial and arcuate [ARC] nuclei). Pharmacological and anatomical data are consistent with γ -aminobutyric acid (GABA)- and glutamate-containing afferents controlling A15 activity in anoestrus, with E₂ inhibiting GABA and stimulating glutamate release at this time of year. Tract tracing demonstrated that A15 efferents project posteriorly to the median eminence and the ARC, suggesting possible direct actions on GnRH terminals or indirect actions via kisspeptin neurones in the ARC to inhibit GnRH in anoestrus. Identification of this neural circuitry sets the stage for development of specific hypotheses for morphological or transmitter/receptor expression changes that would account for seasonal breeding in ewes.

Keywords

anoestrus; dopamine; GABA; glutamate; kisspeptin; oestrogen negative feedback

Reproduction is unique among physiological systems in that it can be shut down completely for prolonged periods. In females, the most common instances of such reversible infertility are the anovulation that occurs prior to puberty, during lactation, and annually in seasonally breeding species. Although the afferent signals inhibiting reproduction vary among these three conditions, the basic changes in hypothalamic function appear to be similar. Specifically two distinct, but not mutually exclusive, alterations in the control of gonadotrophin-releasing hormone (GnRH) secretion have been described. First, there is a change in responsiveness to the negative feedback actions of oestradiol (E₂) so that during

anovulatory periods E_2 exerts a potent inhibition of GnRH secretion that wanes as ovulatory ovarian cycles begin. Second, there is a steroid-independent inhibition of GnRH that is evident as unexpectedly low levels of luteinising hormone (LH) concentrations in ovariectomised (OVX) animals and agonadal girls. Although such instances of physiological infertility occur in all species, the underlying alterations in hypothalamic function remain largely unknown. One exception to this generalization is the ewe, in which a testable hypothesis has been developed for specific hypothalamic alterations to account for the seasonal suppression of reproductive function. In this review, we will first briefly consider the mechanisms by which the external environment controls the timing of ovulation in the ewe, and then describe the neural circuitry that appears to be responsible for seasonal changes in GnRH secretion.

Seasonal breeding in the ewe

Females in most breeds of sheep show distinct seasonal patterns in ovarian function, with ovulatory cycles occurring in the fall and winter (the breeding season) and anovulation in the spring and summer (anoestrous season). This ensures that, under normal conditions, all ewes become pregnant early in the fall and, since pregnancy lasts for five months, lambs are born in the spring when environmental conditions maximize their chances of survival. It has been recognized for over half a century that sheep use photoperiod (i.e., the number of hours of daylight/day) to time breeding and anoestrous seasons (1). This photoperiodic information is perceived by retinal photoreceptors, transmitted as a neural signal via the suprachiasmatic nucleus, the paraventricular nucleus (PVN), and the superior cervical ganglion to the pineal gland, where it is transduced into an endocrine signal, the nightly secretion of melatonin (1,2). Elegant ablation and replacement studies in the early 1980s (3) demonstrated that the duration of elevated melatonin concentrations determines the reproductive status of the ewe. Thus, the daily melatonin pattern can be considered an internal analogue of the external photoperiod. Melatonin appears to act in the premammillary region (PMR) at the posterior end of the medial basal hypothalamus (MBH) (4,5), but does not directly drive changes in reproduction; rather it synchronises an endogenous circannual rhythm in reproductive activity with the external environment (5,6), much as circadian rhythms are synchronised by the 24 hr light-dark cycle. Although considerable information has recently been obtained on how melatonin synchronises another circannual rhythm (prolactin secretion) in sheep (7), the mechanisms by which the circannual rhythm in GnRH secretion is generated and controlled by melatonin remain a mystery.

Our understanding of the basic changes in the hypothalamo-pituitary-ovarian (HPO) axis responsible for seasonal breeding in the ewe developed in parallel with studies on the role of melatonin in mediating the effects of photoperiod. The key observation was the report in 1977 of a dramatic increase in the ability of E_2 to inhibit LH secretion in the anoestrous ewe (8). Subsequent work provided convincing evidence that seasonal changes in response to E_2 negative feedback drove seasonal breeding in ovary-intact ewes and reflected changes in episodic LH secretion (9). During the breeding season, E_2 inhibits LH pulse amplitude but cannot inhibit LH pulse frequency (10–12), while in the anoestrous season, E_2 becomes a potent inhibitor of LH pulse frequency (11,12). A steroid-independent effect of photoperiod is also evident as subtle seasonal changes in LH pulse frequency in untreated OVX ewes (11), but probably plays little role in seasonal breeding in sheep (1). The seasonal changes in the ability of E_2 to inhibit LH pulse frequency pointed to the hypothalamus as the critical site for seasonal changes in the HPO axis (which was confirmed by GnRH measurements, 13–14) and raised the question: what anatomical and functional changes within the hypothalamus are responsible for seasonal changes in E_2 negative feedback? Subsequent

work over the next two decades identified a group of dopaminergic (DA) neurones (A15 neurones) in the retrochiasmatic area of the sheep (15) as central players in this system.

A15 DA neurones

Two sets of studies led to the identification of A15 neurones as important to seasonal changes in E_2 negative feedback. The first of these was our report that pentobarbital anesthesia stimulated episodic LH secretion in ovary-intact anoestrous, but not breeding season, ewes (16). This led to the hypothesis that GnRH pulses were held in check by an inhibitory neural system that was not functional in the breeding season, and subsequent work using classical neuropharmacological approaches implicated DA as the inhibitory neurotransmitter (17–19). The second was the report from Jean-Claude Thiery and collaborators (20) that 6OH-DA lesions of catecholamine neurones in the RCh disrupted the negative feedback actions of E_2 in anoestrous ewes. We confirmed this observation using radiofrequency lesions and demonstrated that these lesions did not affect the negative feedback actions of E_2 in the breeding season (21). Subsequent work using a variety of indices of neural activity, including Fos expression (22), bioactivity of tyrosine hydroxylase (TH) the rate limiting enzyme for DA synthesis (23), and multi-unit electrical activity (24), demonstrated that E_2 stimulates A15 neurones in anoestrous (22–24), but not breeding season (22), ewes. Taken together, these and other (25) studies provide strong evidence that A15 neurones are central players in seasonal breeding in sheep. In anoestrus, E_2 increases their activity which inhibits GnRH pulse frequency, but during the breeding season, A15 neurones do not respond to E_2 , so that this steroid can no longer suppress pulse frequency.

Neural circuitry of seasonal breeding: anatomical inputs to A15 neurones

Although A15 neurones are stimulated by E_2 in anoestrus, they do not contain oestrogen receptor- α (ER α) (26,27), the oestrogen receptor that mediates the negative feedback actions of E_2 in both mice (28) and sheep (29). One can thus infer that ER α -containing neurones send afferent projections to the A15 that stimulate these DA neurones when E_2 levels rise. Studies using microimplants for the local administration of E_2 identified two neural areas where this steroid inhibited LH secretion in anoestrous ewes via a DA system: the retrochiasmatic area (RCh) and the ventomedial preoptic area (vmPOA) (29–31). In contrast, E_2 microimplants in several other areas (e.g., medial or lateral POA, ventromedial hypothalamus) had no effect (30).

To identify anatomical inputs to and outputs from the A15, we microinjected 50–100 nl of 0.1 M phosphate buffer containing a combination of the retrograde tract tracer, cholera toxin- β (0.5% CT β) and the anterograde tract tracer, biotinylated dextran amine (10% BDA), unilaterally into the RCh aimed at the A15 (32). To identify afferents to the A15, tissue was collected two weeks later and processed for dual immunocytochemical (ICC) identification of CT β and ER α (26). In 3 of 6 ewes the injection site was centered on the A15; the smaller (50 nl) injections (n=2) were restricted to this area, while the 100 nl injection extended rostrally into the adjacent RCh (Fig. 1a–b). Since the pattern of retrogradely-labelled cell bodies was similar in these three ewes, data from all of them was included in this analysis. The density of CT β -containing cells was highest (30–50 cells/section) in medial regions, extending from the POA to the PMR (Fig. 1c–f), with particular concentrations in the dorsal and vmPOA, the RCh, the ventromedial (VMN) and arcuate (ARC) nuclei, and the PMR. More scattered retrogradely-labelled cells were observed in the telencephalon (medial septum, diagonal band of Broca, and bed nucleus of the stria terminalis), mesencephalon (periaqueductal gray, reticular formation, and raphe nucleus), and metencephalon (locus coeruleus and parabrachial nucleus). Dual ICC revealed a much more limited distribution of ER α -containing cells projecting to the A15 (Fig. 1c–h), with most of these cells found in

three areas (POA, VMN, and ARC) although scattered dual-labelled cells were also observed in the RCh slightly rostral, dorsal, and medial to the A15. In a follow-up study, CT β alone (50 nl) was injected into the area containing the A15 and a more sensitive immunofluorescence dual ICC procedure used to quantify CT β and CT β +ER α -containing cells in a series of every 4th section (50 μ m thick). A very similar distribution pattern was observed, with 20–40% of CT β -positive neurones in the same three areas also containing ER α (Fig. 1c–h, vertical bars). These results confirm and extend an earlier study (33) that used larger injection volumes (200 nl) of a retrograde tract tracer (fluorogold) resulting in spread to ventral portions of the VMN and the accessory supraoptic nuclei (SON). Because of this larger volume, one can infer that retrogradely-labelled cells observed in that study but not here (which include the SON and PVN, lateral septum, and supramammillary nucleus), likely project to the VMN or accessory SON. In contrast, the distribution of labelled cells in these two studies was very similar for most other POA and hypothalamic areas indicating cells in these areas most likely project to the A15 region.

To confirm the vmPOA as a source of afferent input to the A15, BDA was unilaterally injected (100 nl, 10% solution) into this area and tissue collected two weeks later (BDA injections into the RCh were not attempted because of its close proximity to the A15). In 2 of 6 ewes, BDA was limited to the vmPOA and overlapped with the location of ER α -containing cells (Fig. 2a). In both of these ewes, BDA-labelled fibres and terminals heavily innervated the A15 region (Fig. 2b), with many forming close contacts with TH-positive soma and dendrites. In the 4 ewes with missed injections, no BDA reactivity was observed in the A15. Taken together these results provide direct anatomical support for the functional studies that have implicated the vmPOA and RCh as locations of estrogen-responsive neurones that provide stimulatory input to the A15 during anoestrus.

Neural circuitry of seasonal breeding: functional inputs to A15 neurones

Another important question in characterizing this circuitry is the identification of the neurotransmitters used by A15 afferents. We chose a pharmacological approach to address this question by testing the effects of local administration of selected receptor agonists and antagonists to the A15. Based on ICC studies of cells containing ER α in the sheep, there are six potential neurotransmitters: γ -aminobutyric acid (GABA) (34), glutamate (35), nitric oxide (NO) (36), neurokinin B (NKB) (37), dynorphin (38), and kisspeptin (39). The latter two can be excluded, dynorphin because opioid receptor antagonists do not increase LH secretion in ovary-intact anoestrous ewes (16,40), and kisspeptin based on seasonal changes in its expression (see below). Moreover, local administration of a NKB receptor agonist into the RCh stimulated, rather than inhibited, LH secretion (41), while disruption of NO synthesis in this area had no effect (42). Therefore, we focused our efforts on GABA and glutamate.

Local microinjection of either a GABA_A or a GABA_B receptor agonist into the area containing the A15 produced a dramatic increase in episodic LH secretion in ovary-intact, but not OVX, anoestrous ewes, and confocal microscopic analysis demonstrated that all A15 DA neurons receive GABAergic close contacts (42). Based on these data, we postulated that GABAergic neurones synapse on A15 cells, and that release of GABA is inhibited by E₂ in anoestrous ewes. Thus, there is little GABA release in ovary-intact anoestrous ewes and GABA receptor agonists can activate the unoccupied receptors and inhibit DA release. The fall in E₂ after OVX allows GABA release, which in turn inhibits A15 neural activity and results in the post-castration rise in episodic GnRH and LH secretion. Because of this endogenous GABA release, we propose that GABA receptors are occupied in OVX ewes so the exogenous agonist produces no further effects. The ability of a combination of GABA_A and GABA_B receptor antagonists to inhibit LH secretion when microinjected into the A15

(42) supports the hypothesis that endogenous GABA release is holding the inhibitory A15 neurones in check in OVX ewes.

Because glutamate is an excitatory neurotransmitter we postulated that it would be released in ovary-intact anoestrous ewes to increase DA release from A15 neurones, and therefore we tested the effects of receptor antagonists in these animals. Local administration of either a NMDA or an AMPA receptor antagonist into the region of the A15 increased LH pulse frequency in ovary-intact ewes in a dose-dependent manner (43). These data, together with anatomical evidence for glutamatergic innervation of virtually all A15 neurones (43), support the hypothesis that stimulatory glutamatergic input is driving DA release from A15 neurones in ovary-intact anoestrous ewes. Thus, the negative feedback actions of E₂ in anoestrus results from *both* an inhibition of GABA and stimulation of glutamate release, the combination of which activates A15 neurones and thus inhibit GnRH pulse frequency. One key question currently under investigation is the location of the cell bodies containing GABA or glutamate that provide this input to the A15. There is evidence for ER α -containing GABAergic (34) and glutamatergic (35) neurones in the POA of sheep; whether these neurones, or similar neurones in other areas, project to the A15 remains to be determined.

Neural circuitry of seasonal breeding: outputs from A15 neurones

Although it is clear that DA released from A15 neurones holds GnRH pulse frequency in check in ovary-intact anoestrous ewes, the specific pathway(s) by which this occurs remain to be determined. To identify potential targets of A15 neurones, we examined the distribution of BDA-containing fibres using tissue from the three ewes that received CT β and BDA into the A15 (Fig. 1a). Almost all BDA-positive fibres exiting the A15 coursed ventromedially toward the ARC (Fig. 3a) and median eminence (Fig. 3b) and the highest density of fibres was observed in these areas; only a few scattered BDA-positive fibres were observed in more rostral areas, including the POA. In the median eminence, a majority of fibres were located within the internal zone and continued within the infundibular stalk as it detached from the hypothalamus. However, a significant number of BDA-containing fibres and terminals were also evident in the external zone of the median eminence (Fig. 3b). In the rest of the MBH, BDA-containing projections were primarily observed in areas close to the base of the brain, including the middle (Fig. 3a) and caudal ARC and the premammillary nucleus. Dual ICC for BDA and TH on sections from two ewes who received similar BDA injections demonstrated that some BDA-positive fibres in the ARC (Fig. 3c) and median eminence (Fig. 3d) also contained TH, indicating that they originated from dopaminergic A15 neurones. A previous study, using a larger volume (200 nl) of different anterograde tracers, also found a major A15 projection via the internal zone of the median eminence to the posterior pituitary, but noted that labelled fibres in the external zone were sparse compared to those in the internal zone (44); projections to other areas were not described. The simplest explanation for the quantitative differences in the relative number of projections to the internal and external zones between these two studies is that, as suggested in the earlier report (44), the larger volume of anterograde tracer was picked up by some magnocellular neurones in the accessory SON, whereas our smaller injections did not label projections from these cells.

The observation that A15 neurones project posteriorly is consistent with reports that local administration of either a DA receptor antagonist (45) or a blocker of DA synthesis (46) to the MBH increased LH secretion in anoestrous ewes, whereas similar treatments in the POA had no effect (45). Based on the projections of the A15 that we observed, there are two sites where DA is likely to act to inhibit GnRH: 1) directly in the median eminence and 2) indirectly in the middle-caudal ARC. Three lines of evidence support the possibility of a

direct projection from A15 neurones to GnRH terminals in the median eminence. First, electron microscopic analysis has demonstrated DA synapses onto GnRH terminals in the ovine median eminence (47), although the source of these synapses was not identified. Second, retrograde tracers injected into the median eminence of sheep label cells in the region of the A15 (48). Third, we have observed BDA-positive varicosities in close contact with GnRH terminals in tissue from ewes in which BDA was injected into this region (49).

The other possible site of action of DA released from A15 neurones based on this anatomical study is the middle-caudal ARC (Fig 3A). If A15 neurones inhibit GnRH secretion via this pathway, the most likely candidates to mediate this action are the kisspeptin-containing neurones concentrated in the middle-caudal ARC in ewes (50). There are dramatic seasonal changes in kisspeptin expression in the sheep, with many more kisspeptin-positive cells in the middle-caudal ARC in breeding season, than in anoestrus, ewes (51). This seasonal variation appears to be largely due to an increase in the suppression of both kisspeptin protein and *kiss1* mRNA in these cells by E₂ during anoestrus. Interestingly, there is a corresponding decrease in kisspeptin-containing close contacts onto GnRH neurones in anoestrus, but only in GnRH cells found in the anterior hypothalamic area and MBH (51). Since GnRH cell bodies in the MBH have been specifically linked to episodic GnRH secretion (52), these observations are consistent with the hypothesis that A15 neurones inhibit GnRH pulse frequency in anoestrus by inhibiting kisspeptin stimulation of episodic GnRH release.

Conclusions: possible seasonal changes in A15 neural circuitry

In conclusion, we propose the following model (Fig. 4) for the neural circuitry responsible for E₂ negative feedback in anoestrus ewes. During anoestrus, E₂ acts on ER α -containing neurones in the vmPOA and RCh that project to the A15 neurones. By either increasing glutamate and/or decreasing GABA release from these A15 afferents, E₂ stimulates DA release from A15 neurones. The resulting increase in DA then acts either directly at GnRH terminals in the median eminence, and/or indirectly via kisspeptin neurones in the middle-caudal ARC to inhibit GnRH pulse frequency.

Identification of anatomical and functional inputs to and outputs from A15 neurones, allows for the development of testable hypotheses for alterations in this circuitry that could account for seasonal changes in E₂ negative feedback. First, there could be seasonal changes in responsiveness of ER α -containing neurones in either the vmPOA or RCh. Results from work using Fos as an index of neural activity suggest the vmPOA population is more responsive in anoestrus than in the breeding season (53), which correlates with seasonal changes in the number of neurones containing ER α in the POA (27). Whether these changes in ER α expression occur in GABA or glutamate-producing afferents to the A15 remains to be determined. Alternatively, there could be seasonal changes in afferents from these ER α -containing neurones. This possibility is supported by evidence of an increase in close contacts containing synaptic markers on to A15 neurones in anoestrus (54). Interestingly, there is a similar increase in the number of glutamatergic close contacts on to A15 neurones in anoestrus (43), but no seasonal changes in GABAergic input (42,43). These data raise the possibility that seasonal changes in glutamatergic stimulatory input could account for seasonal differences in the effects of E₂ on A15 neural activity. It should be noted that the lack of seasonal differences in GABAergic synaptic input does not preclude a role for these afferents in the seasonal changes in A15 activity. For example, if ER α expression in GABAergic afferents decreased during the breeding season, E₂ would no longer be able to inhibit GABAergic tone so that A15 neurones would be inactive regardless of circulating E₂ at this time of year. It will thus be important in future work to determine if the expression or effects of GABA or glutamate varies with season.

Although it is clear that seasonal changes in A15 activity are a key component of seasonal breeding, this does not exclude the possibility of seasonal interruption of A15 efferents to either the median eminence or ARC also contributing to the onset of the breeding season. This would be consistent with evidence in the rat where neural plasticity in both the median eminence and ARC occurs in association with changes in reproductive neuroendocrine status (55–57). In Japanese quail, a photoperiodic species, seasonal rearrangements of neuro-glial interactions have been observed in the median eminence (58) but seasonal morphological alterations in either the median eminence or in cells of the ARC (including kisspeptin neurones) have not yet been reported in mammals. There is evidence for seasonal changes in synaptic input (50,59) and glial appositions (59) to GnRH neurones in the MBH of ewes, but these cells are not limited to the ARC.

In summary, seasonal changes in either the morphology or transmitter/receptor expression could contribute to reproductive transitions at multiple levels in the neural circuitry mediating the increased response to E₂ negative feedback in anoestrous ewes. It will be important in future studies to distinguish those changes which are causally related to the onset of seasonal reproductive transitions from those which may occur secondarily as a result of altered patterns of afferent neurotransmitter or GnRH release.

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Figure 1.

Afferents to A15 based on injections of the retrograde tract tracer CT β . Panels a and b illustrate injection sites. Panel a: location of the three injection sites. In two ewes (776, 1127), tracer deposits completely overlapped the location of A15 dopamine neurones (filled circles), in another (2017) the tracer deposit partially overlapped the A15. Panel b: ICC staining of CT β in A15 of ewe 776. Scale bars: 1 mm (a) and 0.2 mm (b). Panels c–F: Drawings of representative sections through the POA (c), rostral (d) and middle (e) and caudal (f) arcuate nucleus (ARC) of ewe 776 summarizing the locations of CT β -labelled cells (black circles) or cells double-labelled with CT β and ER α (red circles), ipsilateral (left side) and contralateral (right side) to A15 injection site (grey, shaded area). Each dot represents 5–10 cells, scale bar: 1 mm. Vertical bars in Panels c–f depict mean ($\pm$ SEM, $n=3$) number of cells containing CT β (open bars) or CT β +ER α (red bars) in areas with major projections to the A15 from a subsequent study using dual immunofluorescence. Panels g and h: dual ICC staining for CT β (brown) and ER α (black) in tissue from the POA (f) and RCh (g). Abbreviations: ac, anterior commissure; fx, fornix; ir, infundibular recess; mt, mammillothalamic tract; OCh, optic chiasm; OVLT, organum vasculosum of the lamina terminalis; VMH, ventromedial hypothalamus; 3V, third ventricle

Figure 2.

Afferents to the A15 from the vmPOA based on injections of the anterograde tracer BDA. Panel a: coronal section through the unilateral BDA injection site on the left (asterisk) also stained for ER α (visible on the right side) illustrating that the injection site overlapped with estrogen-responsive neurones in this area. Panel b: Section through the RCh stained for both BDA (red) and TH (green).

Figure 3.

a, b: Low power photomicrographs showing the distribution of CTb-labelled cells and BDA-labelled fibres in the arcuate nucleus (ARC) (a) and median eminence (me) (b) of ewe 776 (see Figure 1). Arrow in **a** indicates the location of fibres from the A15 coursing medially toward the ARC. Arrow in **b** indicates efferent fibres coursing through the internal zone of the median eminence; in addition, less abundant efferents (e.g., arrowhead) are seen in the external zone. 3v, third ventricle; pt, pars tuberalis of the pituitary. Scale bar = 100 μ m. c, d: Confocal images of sections double-labelled for BDA (red) and tyrosine hydroxylase (TH) (green). Double-labelled fibres (arrows) are seen in the ARC (c) and the external zone of the median eminence (d). Scale bars in c = 20 μ m; d = 10 μ m.

Figure 4.

Model for neural circuitry implicated in the seasonal changes in response to E_2 negative feedback. Estrogen-responsive (blue) neurones in the vmPOA and RCh send stimulatory afferents to the A15 DA (red) neurones, which inhibit GnRH (brown) neurones either at terminals in the median eminence or via kisspeptin (green) neurones in the middle and caudal ARC. Insert depicts tissue from the A15 stained ICC for DA (red), GABAergic (blue), and glutamatergic (green) neural elements, illustrating close contacts of the latter two neurotransmitters on to the A15 neurones (arrows). E_2 is postulated to inhibit GABA release and stimulate glutamate release at these synapses.