Does nitric oxide act in the ventromedial preoptic area to mediate oestrogen negative feedback in the seasonally anoestrous ewe?

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Abstract

Seasonal anoestrus in the ewe results from enhanced oestrogen negative feedback. Recent data have implicated the ventromedial preoptic area (vmPOA) as an important site of oestrogen action. This study addressed whether NO acts within the vmPOA to inhibit LH during seasonal anoestrus. In Experiment 1, microimplants containing *N*- ω -nitro-L-arginine methyl ester (L-NAME, NOS inhibitor), *S*-methyl thiocitrulline (SMTC, neural NOS (nNOS) inhibitor) or empty implants (control) were administered during mid-anoestrus to the vmPOA. L-NAME, but not SMTC, significantly increased LH pulse frequency. For Experiment 2, ewes in late anoestrus were administered 7-nitroindazole (7NI; nNOS inhibitor), L-NAME, SMTC, or empty implants. 7NI, but not L-NAME or SMTC, increased LH pulse frequency. In Experiment 3, the effects of microimplants and microinjections of L-NAME were compared in mid-anoestrus. Microinjections of L-NAME (300 nl at 10 μ g/ μ l) increased LH pulse frequency, but microimplants did not. In late anoestrus, similar microinjections were ineffective. Taken together, the results of Experiments 1–3 suggested that NO inhibition may be stronger during the middle than at the end of seasonal anoestrus. To test this hypothesis, ewes in Experiment 4 received microinjection of L-NAME or vehicle thrice during the non-breeding season; none of the treatments increased LH pulse frequency. These results indicate that NO plays a role in the vmPOA in suppressing LH secretion during seasonal anoestrus because NOS inhibitors were consistently stimulatory when LH pulse frequency was low. However, the inconsistent and modest effects of these inhibitors suggest that NO actions in this area cannot completely account for the effects of inhibitory photoperiod.

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Introduction

In most breeds of sheep, ewes exhibit a seasonal suppression of the reproductive axis that results from the inhibition of pulsatile gonadotrophin-releasing hormone (GnRH) and luteinizing hormone (LH) secretion during the longer days of the spring and summer months (Karsch *et al.* 1993). This annual seasonal infertility is caused by a dramatic enhancement in the responsiveness of GnRH secretory neurones to oestrogen negative feedback (Legan *et al.* 1977, Goodman *et al.* 1982). However, the site at which this effect occurs is not the GnRH neurones themselves since few, if any, GnRH neurones express the α -form of the oestrogen receptor α (ER α ; Herbison *et al.* 1993, Lehman & Karsch 1993). Although recent reports have demonstrated that some GnRH neurones express ERs of the β -subtype and oestrogen-related receptors (Herbison *et al.* 2001,

Hrabovszky *et al.* 2001, Legan & Tsai 2003, Skinner & Dufourny 2005), there is little evidence supporting a role for these receptors in oestrogen negative feedback control of GnRH release (Dorling *et al.* 2003, Hardy *et al.* 2003).

The complete neural pathway by which the response to oestrogen negative feedback changes with season is not yet established, although a considerable body of evidence has implicated two cell groups: 1) ER α -containing neurones in the ventromedial preoptic area (vmPOA) near the organum vasculosum laminae terminalis (OVLT; Stefanovic *et al.* 2000, Anderson *et al.* 2001) and 2) dopaminergic cells of the A15 cell group located within retrochiasmatic area (RCh; Meyer & Goodman 1985, Thiery *et al.* 1989, Havern *et al.* 1994, Lehman *et al.* 1996, Goodman *et al.* 2000). Because A15 neurones do not contain ERs (Lehman &

Karsch 1993, Skinner & Herbison 1997), oestrogen likely influences A15 neurones through afferent inputs from areas such as the vmPOA. Neurones in the vmPOA are stimulated by systemic oestrogen treatment in non-breeding, but not breeding, season ewes (Stefanovic *et al.* 2000) and the expression of ER α in the vmPOA increases in anoestrus (Skinner & Herbison 1997). In addition, localised administration of oestrogen directly to the vmPOA suppresses LH pulse frequency during seasonal anoestrus, but not during the breeding season (Anderson *et al.* 2001).

Some of the oestrogen-responsive neurones in the vmPOA project to the A15, but others do not (Lehman *et al.* 2002). The latter may thus function as important local interneurones, but the phenotype of these neurones remains to be determined. One neurotransmitter that may be produced by these cells and inhibit GnRH release is NO. In the sheep, a subset of oestrogen-responsive neurones within the POA contains NOS (Dufourny & Skinner 2002) and this neurotransmitter has been implicated in the feedback actions of oestrogen in the rat (Brann & Mahesh 1997, Herbison 1998) and other species (Honaramooz *et al.* 1999). Our studies tested the hypothesis that actions of NO are important in the vmPOA for the inhibition of LH secretion that occurs during seasonal anoestrus using local administration of drugs that block the synthesis of NO by inhibiting NOS or neural NOS (nNOS).

Materials and Methods

General

Mature, ovary-intact, seasonally anoestrous (verified by undetectable progesterone in jugular blood samples) black-faced ewes of predominantly Suffolk breeding were used for all experiments. In our flock, anoestrus begins in early March and ends in the last week of August. Ewes were moved to an indoor facility at least 3 days prior to neurosurgery and maintained there for the remainder of the study. This facility had artificial lighting adjusted to natural day length and temperature fluctuated between 15 and 30 °C. *Ad libitum* access to water and a maintenance level diet of alfalfa pellets, and corn was provided daily.

Surgical procedures

Surgical placement of guide cannulae directed to the vmPOA was performed as previously described (Havern *et al.* 1994, Coolen *et al.* 1999, Anderson *et al.* 2001) using sterile techniques with ewes under halothane (~2%) anaesthesia in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). After the top of the skull was exposed, a 20 mm wide $\times$ 30 mm long hole, centred about 10 mm rostral to the bregma, was drilled and punched in the skull and the superior sagittal sinus was

ligated. The left lateral ventricle was temporarily cannulated by lowering a bevelled 18 gauge stainless steel tube placed just rostral to bregma and 4 mm lateral to midline, and radio-opaque dye (iohexol, Omnipaque 350; Winthrop, New York, NY, USA) was injected (2 ml over 1 min) to visualise the ventricles using X-ray radiography. Based upon lateral and coronal X-ray images, bilateral 18 gauge stainless steel guide tubes with the end bevelled to a point (length: 60 mm) were lowered to a position 2 mm dorsal to the target sites for microimplants (target site: 1.5 mm lateral to midline, 2 mm dorsal to supraoptic recess of third ventricle, at the rostral point of this recess in the AP plane). After positioning, the guide tubes were blocked with 22 gauge wire stylets, the lateral cannula was removed, and the exposed brain was covered with gelfoam and a fine nylon mesh. Dental acrylic was applied over the fine mesh and around stainless steel cranial screws inserted for anchorage. The upper portion of a 20 cc plastic vial with a screwable lid was cemented in place with dental acrylic to protect the protruding guide tubes and the skin was sutured around this apparatus (Anderson *et al.* 2001). On the day prior to surgery, 20 mg dexamethasone (i.m.) and 8 ml penicillin (s.c.) were administered. Dexamethasone (10 mg), penicillin (8 ml) and atropine (15 mg; s.c.) were given immediately prior to surgery. A post-surgical analgesic (flunixin meglumine; 100 mg) was administered while the animals were recovering from anaesthesia. Dexamethasone was administered in decreasing daily doses ending 3 days afterwards with 2 mg, and penicillin (8 ml) was also injected daily during this time period. Animals were given at least 2 weeks to recover from neurosurgery before experimental treatments.

Administration of drugs

The non-specific NOS inhibitor N ω -nitro-L-arginine methyl ester (L-NAME), and two nNOS-specific inhibitors, S-methyl thiocitrulline (SMTC) and 7-nitroindazole (7NI), were obtained from Sigma. Drugs were delivered via the insertion of bilateral microimplants (Experiments 1, 2 and 3) or via bilateral microinjections (L-NAME only, Experiments 3 and 4). Microimplants consisted of sterile 22 gauge blunt-ended stainless steel tubes cut to extend into neural tissue 2 mm beyond the 18 gauge guide tubes. The ends of the microimplant tubes were tamped at least 50 times in crystalline drug, leaving the lumen tightly packed with ~250 μ g drug, and the outside cleaned with sterile gauze. Empty microimplants were used for control treatments. Microinjections (300 nl of 10 μ g/ μ l L-NAME or sterile water) were performed using 1 μ l Hamilton syringes with a fixed 22 gauge blunt-ended needle that extended 2 mm beyond the guide tube into neural tissue with the injection time extending <1 min.

General experimental protocol

Peripheral blood samples (3 ml) were collected by jugular venipuncture at 12-min intervals, a frequency of blood collection based on previous experience that allows for easy identification of LH pulses (Goodman & Karsch 1980, Goodman *et al.* 1995). Samples were collected from 36 min before to 4 h after insertion of microimplants or microinjections. This sampling and treatment procedure has provided a reliable model for testing the effects of localised drug administration to specific hypothalamic areas via either microimplants (Havern *et al.* 1991, Goodman *et al.* 2004a) or microinjections (Goodman *et al.* 2004b). The number of LH pulses detected in ewes during seasonal anoestrus is low and random (Goodman *et al.* 1982, Havern *et al.* 1991). Thus, a pre-treatment sampling period equivalent to the post-treatment period (4 h) is no more predictive of LH pulses than a 4-h collection period on previous or subsequent days. The appropriate control under these circumstances is the 4 h after insertion of empty implants or microinjections of vehicle to each animal. Thus, all treatments were assigned using either a Latin-square or crossover design (to control for treatment order), so that each animal served as its own control. In the case of microimplants, the implants were replaced with wire stylets after the last blood sample was collected. Blood samples were stored at 4 °C after clotting and serum was harvested from the blood and stored at –20 °C until hormonal analysis via RIA.

Tissue collection and analysis

Histological verification of the implantation sites was performed as described previously on hypothalamic tissue recovered from the animals after killing (Anderson *et al.* 2001). Four percent (4%) paraformaldehyde-fixed coronal sections (50 µm thick) were cut with a freezing microtome, and every fifth section was mounted on microscope slides and stained with cresyl violet for verification of implant location.

Analyses

RIA

Concentration of LH was determined in 50, 100 or 200 µl aliquots of serum by RIA, using a modification of a previously described method (Havern *et al.* 1994, Anderson *et al.* 2001). Values are expressed in terms of the ovine standard, NIH S24. Radioiodinated ovine LH (AFP-8614B, courtesy of A F Parlow, NIDDK) was used as tracer and primary antiserum was AFP-192279 (courtesy of A F Parlow, NIDDK; dilution 1: 2 000 000). The sensitivity (95% confidence interval at 0 ng/ml) averaged 0.6 ng/tube. Intra-assay coefficients of variation (CV) averaged 13.9 and 10.4% respectively, for serum pools displacing radiolabelled LH to ~36 and 57% of the

total bound, and inter-assay CVs were 14.9 and 15.1% for the same serum pools.

Data analysis

A pulse of LH was defined as previously described (Anderson *et al.* 2001). Significant effects of treatment with the NOS inhibitors on the number of LH pulses detected and mean LH concentrations were identified using Wilcoxin signed rank tests and one-tailed paired Student's *t*-tests for each drug when compared with controls respectively. Data are presented as mean $\pm$ S.E.M. LH pulses/4 h and mean $\pm$ S.E.M. LH concentrations (ng/ml). Amplitude of LH pulses was not compared because the low frequency of pulses observed during control collection periods precluded statistical analysis.

Results and discussion

Microimplant location

Microimplant locations for all experiments are shown in Fig. 1. A similar distribution of the placements was observed for the four experiments (data not shown). Only data from animals with placements known to be correct are included in the analyses (Experiment 1: 6 of the 10 ewes; Experiment 2: 8 of 12 ewes; Experiment 3: 6 of 10 ewes; Experiment 4, 5 of the 8 ewes).

Experiment 1: does NOS inhibition within the vmPOA increase LH pulse frequency during seasonal anoestrus in ewes?

In May 2001, guide tubes were surgically implanted and the ten ewes were then treated during the middle of the non-breeding season (mid-June–early July) with microimplants containing: 1) L-NAME, 2) SMTC or 3) empty microimplants (control) in a Latin-square design with at least 6 days between treatments. No treatments produced obvious behavioural effects. Representative LH profiles are presented in Fig. 2A–C. As shown in Fig. 2B and D, treatment of seasonally anoestrous ewes with L-NAME caused a significant increase in the mean number of LH pulses (1.5 ± 0.3 pulses/4 h for L-NAME versus 0.2 ± 0.2 pulses/4 h for controls; $P=0.02$), with all six ewes showing more LH pulses in the treated than control periods. Following treatment with SMTC three of six ewes had more LH pulses than following control treatments, but the LH pulse frequency for this group ($1.0 \pm 0.6/4$ h), was not statistically different from controls ($P=0.12$). None of these treatments significantly affected mean LH concentrations (Fig. 2E), because the majority of pulses were of low amplitude and one ewe had anomalously high non-episodic LH concentrations (mean of 4.7 ng/ml) following her control treatment.

The stimulatory effects of L-NAME on the number of pulses raised the possibility that NO contributes to the

Figure 1 Placements for the implants/injections directed to the vmPOA for all experiments. Correct placements of the guide tubes are indicated by a closed square, incorrect placements (too anterior or through the base of the POA) are indicated by a black cross and unilateral placements are indicated by an open circle. Each symbol represents the average placement of bilateral implants/injections. Histological preparations for four animals were not available. AC, anterior commissure; DBB, diagonal band of Broca; FX, fornix; LS, lateral septum; mPOA, medial preoptic area; MS, medial septum; oc, optic chiasm; OVLT, organum vasculosum laminae terminalis and SCN, suprachiasmatic nucleus.

negative feedback action of oestradiol. However, because the more specific nNOS inhibitor SMTC was without effect, we repeated this experiment and included another nNOS inhibitor, 7NI.

Experiment 2: do NOS inhibitors administered to the vmPOA increase LH pulse frequency in ewes during late seasonal anoestrus?

Because of a delay in ewe availability, guide tubes were implanted in late June, 2002 and ewes ($n=12$) were treated late in anoestrus (late July–early August) with 1) L-NAME, 2) SMTC 3) 7NI or 4) empty microimplants (control). A Latin-square design was used with 2 days between treatments so that we could get all treatments completed before any animals entered the breeding season. In the ewes with correct guide tube placements ($n=8$), 7NI increased the number of LH pulses in five of eight ewes and pulse frequency was significantly ($P<0.05$) higher with 7NI treatment (2.0 ± 0.4 pulses/4 h) than with control treatments (1.0 ± 0.4 pulses/4 h; Fig. 3A). The number of LH pulses was greater in five of eight ewes in response to the other nNOS inhibitor, SMTC, and in four of eight ewes in

response to L-NAME, although neither of these inhibitors significantly increased LH pulse frequency (SMTC: $P=0.09$; L-NAME: $P=0.11$; Fig. 3A). No significant effects on mean LH concentrations were observed (Fig. 3B) in response to any of the NOS inhibitors.

The lack of a response to localised SMTC administration within the vmPOA of anoestrous ewes in these two experiments was somewhat surprising since SMTC is more potent than L-NAME with 10 μ M SMTC having an inhibitory action comparable to that of 100 μ M L-NAME (Narayanan & Griffith 1994). SMTC is very selective for inhibition of nNOS (Furfin *et al.* 1994), the primary isoform of NOS found in the POA (Bhat *et al.* 1996, Ceccatelli *et al.* 1996). However, SMTC also is slow-binding and does not inhibit 100% of the NADPH oxidase activity of nNOS (Furfin *et al.* 1994). Thus, the slow-binding and incomplete inhibition of NADPH oxidase activity by SMTC may mean that sufficient NOS activity within the vmPOA allowed enough NO to remain in this area to prevent a significant increase in LH pulse frequency. Therefore, SMTC was not used in subsequent experiments.

One possible explanation for the inconsistent effects of L-NAME is that the drug may be diffusing a considerable

Figure 2 LH secretory patterns for representative ewes in Experiment 1 treated with microimplants containing nothing (control; A), L-NAME (B), SMTC (C), during the middle of the non-breeding season. Note the change of scale for LH in B. As shown in D, mean ($\pm$ S.E.M.) number of LH pulses/4 h was significantly increased by L-NAME, but mean ($\pm$ S.E.M.) LH concentration was not significantly altered by any treatment (E). * $P < 0.05$.

distance from crystalline microimplants and thus affecting a large number of NOS-containing interneurons with differing effects on LH. A second explanation is that the response to NOS inhibition may have differed with timing of treatment during anoestrus. Therefore, the objectives of Experiment 3 were to: 1) confirm the stimulatory effects of 7NI, 2) to directly compare the response of ovary-intact anoestrous ewes to

microimplants and more localised microinjections and 3) to test the hypothesis that the response to NOS inhibition varies at two different times during the non-breeding season.

Experiment 3A: do microimplants of NOS inhibitors consistently increase LH pulse frequency in anoestrous ewes, and are microinjections more effective than microimplants in stimulating LH secretion?

In May 2003, guide tubes were implanted and in June the effects of microimplants and microinjections were compared. We originally planned to compare microimplants and microinjections of both L-NAME and 7NI, but discovered, in contrast to previous reports (Yip & Krukoff 2002), that the solubility of 7NI in water was too low for local injections. Ewes ($n = 10$, six of which had correct placements of the guide tubes) were first treated in early June with microimplants of 1) L-NAME, 2) 7NI or 3) control using a randomised design with 2–3 days between replicates. Ten days later the effects of microinjecting 300 nl L-NAME (10 $\mu\text{g}/\mu\text{l}$) or vehicle (water) were compared using a crossover design, with 2 days between replicates (duration between treatments were kept short in an attempt to minimise the effects of time during anoestrus). In the first half of this experiment, neither L-NAME nor 7NI had any effect on LH pulse frequency (Fig. 4A) or mean LH concentration (Fig. 4B). In contrast, microinjections of L-NAME in late June significantly ($P = 0.036$) increased LH pulses frequency from 0.7 ± 0.3 pulses/4 h (vehicle-treated) to 1.8 ± 0.5 pulses/4 h (Fig. 4A), and mean LH concentrations ($P = 0.001$; Fig. 4B).

Experiment 3B: is the effect of NOS inhibitors lost late in anoestrus?

To test the hypothesis that the effect of NOS inhibitors is lost late in anoestrus, microinjection of L-NAME or vehicle was repeated in late July–early August 2003 using the same ewes and protocol as in Experiment 3A. LH pulse frequency was slightly higher in vehicle-injected animals (1.2 ± 0.2 pulse/4 h) than during late June (0.7 ± 0.3 pulses/4 h; $P > 0.05$) and microinjection of L-NAME had no effect on the number of LH pulses (Fig. 4A) or mean LH concentrations (Fig. 4B), with only three of six ewes exhibiting more LH pulses after microinjection of L-NAME than of vehicle.

This difference in response to L-NAME raises the possibility that the inhibition of LH by NO may change during seasonal anoestrus. This is further supported by the results of Experiments 1 and 2: L-NAME increased LH pulse frequency during the middle of, but not late in, the non-breeding season. There is some evidence that 'inhibitory tone', or inhibition of LH secretion, is greatest in the middle of the non-breeding season (Ungerfeld *et al.* 2004), and this may account for the changes in LH

Figure 3 In Experiment 2, mean ($\pm$ S.E.M.) number of LH pulses/4 h (A) was increased by 7NI-containing microimplants when compared with empty microimplants (control) during the late non-breeding season. L-NAME and SMTC did not significantly increase the number of LH pulses/4 h. None of the treatments significantly altered mean ($\pm$ S.E.M.) LH concentrations (B). * $P < 0.05$.

Figure 4 In Experiment 3, microimplants containing L-NAME or 7NI did not significantly alter mean ($\pm$ S.E.M.) number of LH pulses/4 h (A) or mean ($\pm$ S.E.M.) LH concentrations (B) when compared with microimplants containing nothing (control). Microinjection of L-NAME significantly increased the number of LH pulses/4 h and LH concentrations in late June, but not late July. * $P < 0.05$, *** $P < 0.001$. This research was supported by USDA 01-35202-10862 and NIH HD17864. Abstracts containing some of these data were presented at the Society for Neuroscience 32nd annual meeting, Orlando, FL and Society for Neuroscience 34th annual meeting, San Diego, CA, USA. All procedures were approved by the West Virginia University Animal Care and Use Committee and follow NIH guidelines for use of animals in research.

pulse frequency in control treatments between Experiments 1 and 2 and at different times within Experiment 3. These results thus raise the possibility that changes in NOS activity could account for at least part of these changes in the depth of anoestrus. Therefore, Experiment 4 was conducted to determine if the response to NOS inhibition is influenced by the timing of treatment within the non-breeding season.

Experiment 4: does response to microinjection of a NOS inhibitor change during the non-breeding season?

Guide tubes were surgically placed in March 2004 ($n=8$) and the effects of bilateral microinjections (300 nl) of L-NAME (10 $\mu\text{g}/\mu\text{l}$) and vehicle (sterile water) tested in mid-April (early anoestrus), mid-June (mid-anoestrus) and late July (late anoestrus). A crossover design was used with 6 days between the replicates

within each experiment. In the five ewes with successful placement, the number of LH pulses during control treatments did not vary significantly among stages of anoestrus (1.6 ± 0.7 pulses/4 h in April; 2.2 ± 0.9 pulses/4 h in June; 1.6 ± 0.4 pulses/4 h in July). Microinjection of L-NAME had no significant effect on episodic LH secretion (1.8 ± 0.7 pulses/4 h in April; 1.2 ± 0.4 pulses/4 h in June; 2.0 ± 0.6 pulses/4 h in July) or mean LH concentrations (data not shown) at any time during anoestrus ($P > 0.05$).

General discussion

This is the first investigation of the possible role of NO in the photoperiod-induced suppression of LH secretion, but the inconsistent effects of NOS inhibitors complicate interpretation of these results. One possible explanation for the lack of consistent effects in this study is that these drugs did not reach a sufficient number of neurones to cause significant changes in LH pulse frequency. However, several lines of evidence suggest that this is not the case. First, microimplants of hormones (Anderson *et al.* 2001, 2003, Hardy *et al.* 2003) and receptor antagonists (Havern *et al.* 1991, Hardy *et al.* 2002) have altered LH secretion when placed in the vmPOA (Anderson *et al.* 2001, 2003) or elsewhere in the ovine hypothalamus. Second, microimplants containing the NOS inhibitors were completely empty after 4 h *in vivo* and L-NAME-containing microimplants were empty after a 10 min *in vitro* incubation in saline (data not shown), indicating that this route of administration would produce maximal local concentrations of these drugs. Finally, the dose of L-NAME (10 $\mu\text{g}/\mu\text{l}$) chosen for microinjections was similar to doses that impaired processes mediated by NO synthesis in the working memory of rats (Ohno *et al.* 1993) and much higher than the 10 ng/ μl dose of N ω -nitro-L-arginine that suppressed LH secretion in ovariectomised rats (Moreno & Franci 2004). Practical considerations precluded a fuller dose-response curve, so we cannot rule out the possibility that lower doses of NOS inhibitors would have stimulated LH secretion, but we are not aware of any studies supporting this type of dose-response curve for NOS inhibition.

Although NOS inhibitors sometimes increased LH pulse frequency, presumably through increasing GnRH pulse frequency, these effects were not consistently observed. Therefore, it seems unlikely that NO plays the central role in mediating oestrogen negative feedback within the vmPOA of anoestrous ewes. However, in all experiments in this study, inhibition of NOS increased LH pulse frequency when control animals had a low frequency of LH pulses (< 1 pulse/4 h). Thus, NO does appear to play a role in the suppression of GnRH and LH during anoestrus. These observations raise the possibility that NO may mediate effects of a system that is superimposed upon the photoperiodic-driven changes in oestrogen negative feedback that occur at the

transitions between seasons. As discussed below, NO could theoretically be responding to any of four potential inhibitors of GnRH: 1) photoperiodic-driven changes in oestrogen negative feedback within anoestrus; 2) episodic increases in oestrogen concentrations; 3) low nutrition and/or 4) stress.

The first possibility is that an increase in NO in the middle of seasonal anoestrus produces a stronger inhibition of LH pulse frequency than observed earlier and later in the non-breeding season. The differences in LH pulse frequencies among controls and the effects of NOS inhibitors in Experiments 1–3 are consistent with this possibility, but the results of Experiment 4, are not. However, the controls in Experiment 4 did not show the slowing of frequency in the middle of anoestrus evident in Experiments 1–3 and one of the ewes showed consistently elevated LH pulse frequencies throughout (2–4 pulses/4 h), skewing the overall mean. Therefore, we are reluctant to completely reject the hypothesis that changes in NO inhibition contribute to a slowing of LH pulse frequency in the middle of anoestrus. We are unaware of any studies to date that have examined seasonal changes in NOS activity in the hypothalamus of a seasonally breeding species.

A second possibility is that NO may be relatively insensitive to oestrogen concentrations so that this system is stimulated only when oestrogen levels peak at the time of an endogenous episode of oestrogen secretion that follows each LH pulse (Scaramuzzi & Baird 1977). Because episodic LH and oestrogen secretion is infrequent and irregular in anoestrus ewes (Yuthasastrakosol *et al.* 1977) this could account for the inconsistency of NOS inhibitors in this study. One test of this hypothesis would be to determine if there is a negative correlation between peak oestrogen concentrations and LH secretion. There are considerable technical problems (e.g. low oestradiol levels and infrequent pulses) to testing this prediction in anoestrous ewes, but no negative correlation was evident between LH secretion and episodic elevations of progesterone or testosterone in luteal phase ewes (Alecozay *et al.* 1988) or rams (Lincoln 1976) respectively.

Third, it is possible that NO mediates the effects of another inhibitory system that is only active sporadically. Two inhibitory systems have been extensively investigated in sheep: undernutrition (l'Anson *et al.* 1991) and stress (Smith *et al.* 2003). Undernutrition can be ruled out because these animals were well nourished and LH secretion in adult ewes is very resistant to malnutrition (Goodman & Inskeep 2006). A variety of stressors dramatically inhibit GnRH and LH secretion (Smith *et al.* 2003, Goodman & Inskeep 2006) in adult ewes, and stressors such as high temperature might act to inhibit LH at some times and not others. Although NOS is stimulated by stress in rats (Kishimoto *et al.* 1996, Kim & Rivier 2000), we know of no evidence directly linking NO to stress-induced inhibition of LH secretion in sheep, however, recently,

glucocorticoid receptors have been found to colocalise with NADPH diaphorase-positive neurones in the preoptic area, arcuate nucleus and ventromedial nucleus of the ewe (Dufourny & Skinner 2002). The high degree of coexistence between NADPHd/NOS activity and the expression of glucocorticoid receptors in sheep suggests a direct control of NO production by corticosteroids and a possible mechanism by which glucocorticoids may inhibit GnRH secretion (Dufourny & Skinner 2002).

Finally, it should be noted that these data do not preclude a role for NO in other aspects of systems controlling seasonal reproduction. For example, since some ER α -containing neurones project beyond the vmPOA to the hypothalamic A15 region (Lehman *et al.* 2002), NO could mediate the stimulatory actions of oestrogen on these dopaminergic neurones. However, preliminary data suggest that local administration of NOS inhibitors to the A15 did not affect LH secretion during anoestrus (Hardy *et al.* 2002). In addition to its actions as a neurotransmitter, NO can also facilitate synaptic plasticity, and act to establish or strengthen synaptic connections (Gally *et al.* 1990). Thus, it is possible that NO could play an important role in the seasonal plasticity that occurs in the neural systems mediating seasonal breeding (Adams *et al.* 2006), an action that would not be evident during seasonal anoestrus after these synapses have become established.

In conclusion, the results of the current experiments suggest a role for NO in the vmPOA in the suppression of GnRH and LH secretion in anoestrous ewes. However, in light of the inconsistent effects of NOS inhibitors during anoestrus, it is unlikely that NO is the critical neurotransmitter mediating the dramatic seasonal changes in oestrogen negative feedback that occur in the spring and fall. The data also raise the possibility that NO may act in the vmPOA to mediate the effects of a system capable of further inhibiting GnRH release beyond that produced seasonally in this species.

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