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Abstract

Background: Loss-of-function mutations in genes encoding kisspeptin or neurokinin B (NKB) or their receptors cause infertility. NKB is coproduced in kisspeptin neurons in the arcuate nucleus (ARC), and these neurons also produce the NKB receptor (NK3R), allowing autosynaptic function. We tested the hypothesis that NKB action in ARC kisspeptin neurons is aligned with increased pulsatile secretion of luteinizing hormone (LH) and/or activation of the estrogen-induced LH surge in ewes. **Methods:** Using in situ hybridization and immunohistochemistry, we examined NKB expression in kisspeptin neurons during the ovine estrous cycle. We infused kisspeptin, senktide (an NK3R agonist), or dynorphin into the lateral ventricle during the luteal phase of the estrous cycle to determine effects on pulsatile LH secretion. Finally, we examined the effect of an NK3R antagonist (MRK-08) in ovariectomized ewes. **Results:** NKB *(Tac3)* mRNA expression in mid-ARC kisspeptin neurons was elevated during the mid-to-late follicular phase of the estrous cycle. The number of NKB-immunoreactive cells and NKB/kisspeptin terminals in the median eminence was similar during the estrous cycle. Kisspeptin and senktide increased LH pulse frequency and mean LH levels. Central MRK-08 infusion eliminated the LH pulses but did not prevent an estrogen-positive feedback on LH secretion. **Conclusions:** NKB expression in ARC kisspeptin neurons is upregulated during the late follicular phase of the estrous cycle, when the pulsatile secretion of gonadotropin-releasing hormone (GnRH)/LH is maximal. When GnRH/LH secretion is minimal, central senktide infusion induces LH secretion, similar to the response to kisspeptin. Although the increase in LH in response to senktide appeared surge-like, we did not observe any change in the surge following NK3R antagonist treatment. We conclude that NKB plays a role in increasing basal GnRH/LH pulsatility in

the follicular phase of the cycle but is not essential for estrogen-induced positive feedback.

Introduction

The pulsatile release of gonadotropin-releasing hormone (GnRH) is regulated throughout the estrous cycle by the negative and positive feedback actions of sex steroids [1--3]. Because GnRH neurons do not express estrogen receptor-α or progesterone receptors [4--6], the feedback effect of these sex steroids must be transmitted through intermediary neurons. There is now overwhelming evidence from a number of species, including humans, to indicate that kisspeptin neurons in the arcuate nucleus (ARC) and the preoptic area are the predominant intermediary elements that transmit sex steroid feedback effects to GnRH neurons [2, 7, 8]. In sheep, kisspeptin cells are located in the dorsolateral preoptic area and ARC [9--11], and the population in the ARC relay negative feedback effects of sex steroids. The ARC kisspeptin cells initiate the positive feedback effect of estradiol in ewes, and the preoptic area kisspeptin cells potentiate the effect to cause a preovulatory surge in GnRH/luteinizing hormone (LH) [9, 12--14].

Neurokinin B (NKB) is a peptide belonging to the tachykinin family that has been shown to play a critical role in reproduction. Mutations in the NKB gene *(TAC3)*, or its receptor NK3R *(TACR3)*, cause hypogonadotropic hypogonadism in humans [15, 16]. In sheep [17--19], and in other species [20, 21], NKB and dynorphin are coproduced in ARC kisspeptin neurons; these peptides have been shown to mediate the negative feedback actions of progesterone [18]. The acronym ‘KNDy’ (kisspeptin/NKB/dynorphin) has been applied to these neurons. The KNDy neurons also express NK3R and dynorphin receptor (the κ-opioid receptor) [21], allowing for autoregulation of the cells by NKB and dynorphin [21--23]. In sheep and mice, KNDy cells project to the median eminence (ME), and kisspeptin can elicit a GnRH release at this level [24, 25]. NKB, or the NK3R agonist senktide, stimulates LH secretion in a number of species [26--30], although some inhibitory action has also been reported in rats [27, 31, 32]. In female sheep, however, intracerebroventricular infusion of senktide causes the release of LH during the follicular

phase of the estrous cycle [26], with plasma levels reaching values similar to those seen during the preovulatory surge.

Studies on the hierarchy of action of NKB and kisspeptin to stimulate GnRH/LH secretion lead to the notion that NKB acts upstream of kisspeptin to potentially modulate its expression [27, 29, 30, 33]. As for kisspeptin expression in KNDy cells, NKB expression is suppressed by chronic estradiol [27, 34, 35] and elevated in ovariectomized (OVX) animals [21] as well as in menopausal women [36, 37]. Thus, NKB action on KNDy neurons could be part of the mechanism for sex steroid feedback regulation of the reproductive axis. Most recently, Young et al. [38] studied patients with deficiencies in NKB signaling and showed that gonadotropin secretion could be corrected with kisspeptin treatment, providing evidence that NKB autoregulation occurs in humans. In addition, little is known about the functional role of dynorphin in this system, although this peptide reduces pulsatile LH secretion in goats [30] and appears to be integral to progesterone negative feedback [18].

The working model for the positive feedback effect of estradiol to elicit the preovulatory surge in LH secretion in ewes is as follows. Estradiol acts in the mediobasal hypothalamus (not the preoptic area) to activate the positive feedback mechanism [39]. During the estrous cycle, critical levels of circulating estradiol are reached during the follicular phase, and this activates neurons in the ARC and the ventromedial hypothalamic nucleus [40, 41]. In particular, estradiol causes acute activation of KNDy neurons [13], leading to the GnRH surge, with a time delay [40]. At the time of the GnRH/LH surge, positive feedback is facilitated by recruitment of the kisspeptin cells in the preoptic area [12]. The extent to which NKB is involved in the positive feedback effect is not clear. To investigate this in ewes, the expression of NKB and dynorphin in KNDy neurons was measured across different phases of the estrous cycle. We also measured levels of NKB and kisspeptin protein within terminals of the ME. Finally, we performed intracerebroventricular infusions of kisspeptin, senktide, dynorphin, and the NK3R antagonist MRK-08 to ascertain effects on pulsatile and surge-like LH secretion.

Materials and Methods

Animals

Corriedale ewes of similar age (5--6 years) and weight (64.3 ± 1.2 kg) were maintained at the Monash University Sheep Facility (Werribee, Vic., Australia), and the

experiments were carried out in accordance with the Code of Practice for the Care and Use of Animals for Experimental Purposes provided by the National Health and Medical Research Council/Commonwealth Scientific and Industrial Research Organisation/Australian Animal Commission. The work was approved by the Animal Ethics Committee of the School of Biomedical Sciences, Monash University.

Peptides

Kisspeptin, senktide, and dynorphin A were purchased from Phoenix Pharmaceuticals Ltd. (Belmont, Calif., USA). Kisspeptin peptide YNWNSFGLRY-NH2 corresponding to the murine C-terminal Kiss1 decapeptide (110–119)-NH2 is identical to the C-terminal region of ovine peptide. The use of senktide (succinyl-DFmeFGLM-NH2), an NK3R agonist, has been characterized previously in rodents [31] and sheep [26]. The use of dynorphin A (YGGFLRRIRPKLK) has been characterized in rats [42] and goats [30]. The NK3R antagonist (MRK-08) was developed and tested as described previously [43, 44].

Experiment 1: Expression of NKB in KNDy neurons

Animals and Tissue Collection

Estrous cycles were synchronized in 17 ewes by an intramuscular injection of a synthetic prostaglandin (Estrumate, 125 µg; Pitman-Moore, Sydney, N.S.W., Australia). Groups of ewes were killed by an intravenous overdose of sodium pentobarbital (Lethabarb; Virbarc, Peakhurst, N.S.W., Australia) during the luteal (n = 4), mid-follicular (n = 4), late follicular (n = 6), or estrous phase (n = 3), and their brains were perfused with paraformaldehyde as described previously [9, 13]. The estrous cycle stage was confirmed by examination of ovarian morphology and plasma progesterone (luteal phase), as well as LH (>5 ng/ml, estrous) (data not shown).

Hypothalami were dissected out of the brains and postfixed for 24 h at 4°C, then placed in phosphate buffer containing 30% sucrose for 7 days. The hypothalamic blocks were then frozen and stored at --20°C. Cryostat sections were cut at 30 µm and stored in cryoprotectant solution (containing 2% paraformaldehyde for in situ hybridization) at --20 C.

Double-Label in situ Hybridization

A 262-bp antisense riboprobe for NKB (*Tac3:* gene bank accession No. XM_004006562.1) was prepared by the standard methodology [45]. Fragments were used as templates to synthesize ^{35}S-labeled antisense riboprobes using SP^{6} MEGAscript high-yield transcription kits (Ambion, Austin, Tex., USA). The *Kiss1*-specific template spanned bases 1–357 of the partial ovine cDNA sequence (GenBank accession No. DQ059506). Digoxigenin (DIG)-labeled antisense *Kiss1* riboprobes were transcribed with a MEGAscript SP^{6} transcription kit (Ambion) and DIG-11-UTP (Roche, Indianapolis, Ind., USA) according to the manufacturers' protocol.

Double-label in situ hybridization was performed as described previously [46, 47], using 3 sections from the ARC of each ewe, representing the rostral, middle, and caudal regions (fig. 1a). Briefly, caudal sections were chosen 150 µm from the mammillary recess, and subsequent middle and rostral sections were selected appropriately [48]. The sections were hybridized with the DIG-*Kiss1* riboprobe [47] and the ^{35}S-labeled *Tac3* riboprobes (5 × 10^{6} cpm/ml) at 53°C overnight. After posthybridization washes with descending concentrations of citrate acid and NaCl (SSC), sections were rinsed twice in Tris-buffered saline (0.1 M Tris-HCl, 0.9% NaCl, pH 7.4). The *Kiss1* neurons were revealed with an alkaline phosphatase-conjugated sheep anti-DIG antibody (dilution 1:1,000; Roche) and a colorimetric solution of nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate salts (Roche). The ^{35}S signal was revealed in NKB neurons, as silver grain staining. The sections were coated with 3% Parlodion in isoamyl acetate, dried, dipped in photographic emulsion (Ilford Imaging, Melbourne, Vic., Australia), and left at 4°C for 1 week. Grain-counting software (Image-Pro plus; Media Cybernetics, Silver Spring, Md., USA) was used to count the number of *Tac3* mRNA silver grains over each *Kiss1* cell under darkfield illumination. The signal-to-noise ratio was set at 3× background.

Immunohistochemistry

Sections representing the rostral, middle, and caudal regions of the ARC/ME (fig. 1a) were processed for immunohistochemistry to visualize NKB and kisspeptin peptides as previously described [25]. In brief, antigen retrieval was performed in 0.1 M citric acid (pH 6.0), blocking with normal goat serum, and a guinea pig anti-NKB antibody (1:1,000; gift of Professor Philippe Ciofi, Neurocenter Magendie, Bordeaux, France) and a rabbit anti-kisspeptin antibody (1:2,000, AC566; gift of Dr. Alain Caraty, INRA, Nouzilly, France)

were used. Immunoreactivity was detected with Alexa Fluor 569 anti-guinea pig and Alexa Fluor 488 anti-rabbit secondary antibodies (Vector, Burlingame, Calif., USA). Single- and double-labeled NKB-immunoreactive (ir) and kisspeptin-ir neurons were visualized under the appropriate fluorescence and counted by a single observer using Z-stack microscopy (Zeiss Apotome microscope; Carl Zeiss Inc., North Ryde, N.S.W., Australia) to determine the percent colocalization of the two peptides. The percentage of kisspeptin-ir terminals colocalized with NKB-ir terminals within the ME was determined by Manders' coefficients using JACoP [49] with ImageJ 1.43u (National Institutes of Health, Bethesda, Md., USA).

Experiment 2: Effect of Kisspeptin, Senktide, or Dynorphin on LH Secretion in Luteal-Phase Ewes

Lateral ventricular (LV) cannulae were inserted into normal ewes as described previously [50]. Two weeks after LV surgery, their estrous cycles were synchronized as above. During the mid-luteal phase, one external jugular vein was cannulated for blood sampling, and the animals were housed in single pens. The following day, infusion pumps (Graseby MS16A; Graseby Medical Ltd., Gold Coast, Qld., Australia) were connected to the LV cannulae, and blood samples (5 ml) were collected every 10 min for 9 h. After 3 h, the ewes received a 4-hour continuous infusion of either kisspeptin (404 µg/h, with an initial 20-µg loading dose; n = 6), senktide (404 µg/h, with an initial 20-µg loading dose; n = 5), dynorphin (404 µg/h, with an initial 20-µg loading dose; n = 5), or vehicle (artificial cerebrospinal fluid, aCSF; n = 6) into the LV (200 µl/h) [25]. The dose of peptide was determined in preliminary experiments (data not shown) and previous data [26, 31, 42]. Blood sampling continued for a further 2 h after the infusion. Plasma was harvested and frozen at --20°C until assayed.

Experiment 3: Effect of NK3R Antagonist on LH Pulses and the LH Surge in OVX Ewes

The ewes were bilaterally OVX; LV cannulae were inserted, and the ewes were prepared for blood sampling as described previously [25]. Infusion pumps were connected to the LV cannulae, and blood samples (5 ml) were collected every 10 min. After 3 h, the ewes received either NK3R antagonist (3-hour continuous infusion of 80 nmol/h; n = 3) or vehicle (aCSF with 5% DMSO; n = 3) into the LV (200 µl/h). After infusion, the LV lines remained in place as blood sampling continued for 3 h. Plasma was harvested immediately and frozen at --20°C until assayed.

To determine the critical role of kisspeptin signaling in mediating estradiol-positive feedback and generating the LH surge, we administered the NK3R antagonist to OVX ewes under an estradiol-induced GnRH/LH surge model [51]. The ewes were prepared as described above and received an intramuscular injection of 50 µg estradiol benzoate (EB; Intervet, Wyong, N.S.W., Australia) in 1 ml peanut oil. Blood sampling (5 ml at 10-min intervals) took place for 30 min prior to EB injection, and recommenced 12 h later for 7 h and then every 30 min for a further 9 h. At 10 h after EB injection, the ewes received LV infusions (200 µl/h) of NK3R antagonist (14-hour continuous infusion of 80 nmol/h; n = 5) or vehicle (aCSF with 5% DMSO; n = 5). This time frame for treatment was chosen to begin at least 4 h before the predicted LH surge, which is known to occur between 14 and 18 h after EB treatment [51]. Plasma was harvested immediately and frozen at --20 C until assayed.

LH Radioimmunoassay

Plasma LH concentrations were measured in duplicate, using the method of Lee et al. [52]. The assay sensitivity was 0.1 ng/ml, and the intra-assay coefficient of variation was <10% over the range of 0.6–15 ng/ml. LH pulse analysis (frequency and amplitude) was performed based on the method described previously [25]. For experiments 2 and 3, the mean LH concentration, LH pulse frequency, and LH pulse amplitude were determined in the time periods before (0--180 min), during (180--420 and 180--360 min, respectively), and after (420--540 and 360--540 min, respectively) the infusion. For experiment 3, LH surges were taken to have begun when a clearly evident monophasic rise in plasma LH levels occurred.

Statistical Analysis

All grouped data are presented as means (± SEM). For experiment 1, statistical analyses were initially conducted, using two-way ANOVA. For experiments 2 and 3, grouped data were initially examined, using repeated-measures ANOVA. Where appropriate, a subsequent one-way ANOVA using Tukey's multiple comparison post hoc test was conducted. Surge amplitude and time to peak were examined using Student's t tests. Differences were considered significant at $p < 0.05$.

Results

Colocalization of NKB (Tac3) and Kiss1 mRNA in the ARC

Tac3 mRNA-containing neurons were concentrated in the ARC of all animals examined (fig. 1b). Virtually all DIG-labeled *Kiss1* neurons in the ARC also expressed *Tac3* mRNA. On the other hand, only 55% of *Tac3* mRNA-expressing neurons expressed *Kiss1* mRNA (a total of 4,061 *Kiss1*+ *Tac3* neurons and 7,335 NKB neurons counted; fig. 1c), indicating the presence of cells that express *Tac3* mRNA but not *Kiss1* and/or the difference in the degree of sensitivity of the two detection methods used (DIG labeling is less sensitive than radioactive labeling). The percentage of double-labeled *Kiss1*– *Tac3* neurons did not change across the estrous cycle (data not shown).

Expression of Tac3 mRNA in KNDy Neurons across the Estrous Cycle

Tac3 expression in *Kiss1* neurons was higher ($p < 0.05$) in the late follicular phase of the cycle than in the luteal phase, but this result was confined to the middle ARC (fig. 2a). The absolute number of *Tac3* mRNA-expressing neurons was also significantly ($p < 0.05$) higher in the middle ARC during the late follicular phase than in the luteal phase (fig. 2b).

Expression of NKB and Kisspeptin across the Estrous Cycle

To examine changes in NKB expression at the protein level across the estrous cycle, we quantified the number of NKB-ir neurons and the colocalization with kisspeptin-ir across the ARC. Virtually all kisspeptin-ir neurons were colocalized with NKB-ir neurons, and virtually all NKB-ir neurons were colocalized with kisspeptin-ir neurons (fig. 3a). In general, the total number of NKB-ir neurons appeared to be lower than that of NKB mRNA-containing neurons (4,038 vs. 7,335 cells). There was no significant difference in the number of NKB-/kisspeptin-ir neurons in the ARC across the estrous cycle (fig. 3b).

Colocalization of NKB-ir and Kisspeptin-ir Terminals in the ME throughout the Estrous Cycle

NKB-ir and kisseptin-ir terminal fibers were abundant throughout the ME as reported previously [25]. Overall, there was a 33% colocalization of NKB-ir and kisseptin-ir neurons in these fibers (fig. 4a, b), again similar to earlier data [25]. Across the estrous cycle, no significant difference was observed in the percent colocalization of NKB-ir and kisseptin-ir fibers (fig. 4b). Neither was there any difference in the density of NKB-ir (fig. 4c) or kisspeptin-ir fibers in the ME (fig. 4d).

Stimulation of LH Secretion by Central Infusion of Kisspeptin and Senktide

Kisspeptin infusion into the lateral ventricle increased plasma LH concentrations (fig. 5a, b) with an increase in LH pulse frequency ($p < 0.001$; fig. 5c) in luteal-phase ewes. Central infusion of the NK3R agonist senktide also led to an increase in plasma LH levels, again with an increase ($p < 0.001$) in pulse frequency (fig. 6c); however, central infusion of dynorphin to into luteal-phase ewes had no discernable effect on either pulse frequency (fig. 6c) or pulse amplitude (fig. 6d).

Inhibition of LH Secretion by Central Infusion of the NK3R Antagonist MRK-08

MRK-08 showed clear antagonistic actions on LH levels in OVX ewes. Pulsatile secretory episodes of LH were evident in aCSF control ewes and treated ewes before but not during/after NK3R antagonist treatment (fig. 7a, b). Only a single pulse was detected in one animal during the 3-hour infusion of the antagonist, and pulses were undetectable in the 3 h following infusion. As a result, both pulse frequency and pulse amplitude were significantly reduced during and after infusion in NK3R antagonist-treated ewes ($p < 0.05$; fig. 7c, d).

To determine the importance of NKB signaling in transmitting the estrogen-positive feedback signal to induce the LH surge, a central infusion of MRK-08 was applied under an estradiol-induced surge protocol. LH surges occurred in all NK3R antagonist-treated ewes and controls (fig. 8a, b). The surge amplitude (fig. 8c) and onset (control: 16.5 ± 2.5 h; MRK-08: 16.9 ± 0.4 h) were unchanged. The time from surge onset to surge peak was significantly longer ($p < 0.05$) in NK3R antagonist-treated ewes (fig. 8d).

Discussion

Here we show that NKB mRNA expression is upregulated in kisspeptin neurons in the middle ARC during the late follicular phase of the estrous cycle in the ewe. Central infusion of NK3R agonist into luteal-phase sheep increased LH pulses and produced heightened levels of LH in plasma, the response being similar to that obtained with kisspeptin infusion. Alternatively, infusion of a potent NK3R antagonist eliminated pulsatile LH secretion in OVX ewes but did not prevent the estradiol-induced LH surge. These data support the hypothesis that NKB signaling is integral to basal regulation of GnRH secretion but is not essential for the positive feedback effect of estradiol that is initiated by KNDy cells in the ARC.

Our data are consistent with the notion that NKB is involved in the regulation of ARC kisspeptin neurons and, in turn, the pulsatile secretion of GnRH/LH. NK3 receptors are expressed in these neurons, and the popular model is that NKB acts in an autoregulatory way to synchronize the output of kisspeptin release [20, 21, 23]; this is summarized in a recent review [53], emphasizing that NKB acts upstream of kisspeptin [27, 29, 30, 33]. Notably, the stimulatory effect of senktide on LH is abolished in *Kiss1r* knockout mice [33], and kisspeptin infusion restores LH pulses in human patients with loss-of-function mutations in NKB or its receptor. Collectively, the available data suggest that kisspeptin can stimulate pulsatile GnRH secretion independently of NKB or its receptor, but NKB acts upstream of kisspeptin [38].

NKB appears to be essential to the central control of reproduction in both humans and mice [15, 16, 54], but the role this peptide plays as a mediator of sex steroid feedback regulation during the estrous cycle is not completely understood. Studies in mice, rats, sheep, and primates have shown that NKB gene expression is reduced by estrogen [28, 34, 35] and elevated by ovariectomy in animals and in postmenopausal women [21, 36, 37]. This is compelling evidence that NKB function is fundamental to negative feedback regulation of pulsatile GnRH secretion. The present study showed that the number of NKB (*Tac3* mRNA)-/kisspeptin-expressing cells in the middle ARC increases during the follicular phase, which is concomitant with increased pulsatile GnRH/LH secretion at this stage of the cycle. Interestingly, this change is not consistent with data showing that estradiol inhibits *Tac3* mRNA. Nonetheless, it seems most likely that the increased kisspeptin expression at this time of the cycle is due, at least in part, to the positive stimulus of NKB production at the shift from negative to positive feedback. Consistent with this notion, LV infusion of senktide into luteal-phase ewes increased the detectable pulsatile secretion of LH in a manner similar to kisspeptin infusion. Moreover, infusion of an NK3R antagonist into OVX ewes with free-running pulsatile secretion of GnRH/LH had a powerful suppressive effect. It should also be noted that the response to the constant infusion of kisspeptin (increased LH pulse frequency) indicates that kisspeptin neurons (or kisspeptin ‘pulses’) are unlikely to be functioning predominantly as the ‘GnRH pulse generator’ per se [55] but rather as a modulator of the GnRH pulses intrinsic to GnRH neurons [56, 57].

While it is reasonable to conclude that NKB and dynorphin in kisspeptin neurons are involved in the negative feedback mechanism, the question arises as to whether the same may be true for the positive feedback effect of estrogen that is initiated by these

cells [9, 13]. A recent study in ewes has shown that infusion of the NK3R agonist senktide into the third ventricle produced a 'surge-like' secretion of LH [26]. Our present data show that similar responses may be obtained in luteal-phase ewes, although the mathematical analysis indicated an increased pulsatile secretion in response to senktide, but this could be construed as a 'surge-like' LH response – although the magnitude appears much lower than the endogenous surge. On the other hand, we observed an increased NKB mRNA content in *Kiss1* neurons in the middle ARC during the late follicular phase – not in the caudal ARC, where the cells thought to initiate positive feedback are located [9, 13]. Most importantly, we saw no effect of an NK3R antagonist on the onset or amplitude of an estradiol-induced LH surge. Accordingly, it does not appear that NKB signaling is vital to the mechanism by which estrogen elicits the GnRH/LH surge. We did, however, observe a delay in the onset-to-peak time of the surge; thus NKB may play some minor role in the positive feedback response. Consistent with this, a study by Billings et al. [26] showed that direct NK3R antagonist administration to the retrochiasmatic area reduced the amplitude of the LH surge in ewes. While we are confident that our antagonist reached the ARC, blocking NKB signaling in KNDy cells, we cannot confirm whether our antagonist reached additional sites of NKB action. Nor can we confirm that MRK-08 was effective prior to the *activational* stage of estradiol-positive feedback. Thus, a role of NKB in the LH surge cannot be completely ruled out.

In addition to the KNDy neuron autoregulation, alternative pathways for NKB regulation of GnRH/LH secretion have been proposed. As stated above, it has been suggested that NKB can act in the retrochiasmatic area to stimulate LH secretion [26], but the neural pathway for this requires definition. Moreover, kisspeptin may stimulate GnRH release at the level of the neurosecretory terminals in the ME [24, 25], and it is possible that NKB directly acts upon the GnRH terminals. Highly abundant NKB-ir/kisspeptin-ir terminals are found in the external zone of the ME, and they are in close apposition to GnRH terminals [14, 20, 25, 58]. NK3R is detectable by immunohistochemistry on GnRH terminals in the rat ME, but the NKB cells do not appear to be hypophysiotropic in this species, because they do not take up intraperitoneally injected aminostilbamidine [59]. Other studies in male mice and in ewes showed no receptor expression in GnRH neurons [22, 28], so a direct action on GnRH neurons and/or terminals seems unlikely. Our data showing a lack of change in the relative intensity of NKB fiber labeling over the estrous cycle further argues that there is a lack of cyclical regulation of GnRH secretion by NKB at this level. This is consistent with our

observation that there is no cyclical change in the intensity of kisspeptin terminals in the ME. Interestingly, quantitative assessment of kisspeptin/NKB fibers in the ME indicated only a 30% overlap, which is consistent with our previous data [25] and may reflect an underestimate of absolute colocalization of fibers because immunostaining is rarely continuous along the entire fiber length. On the other hand, some NKB-expressing cells in the ARC do not coexpress kisspeptin, indicating a population of NKB cells that are not KNDy cells (fig. 1). It must, however, be acknowledged that our technique of quantifying the immunoreactive signal in the ME (and in the ARC) may lack the sensitivity to detect change attributed to cyclic variation. The absence of any increase in kisspeptin terminal content is consistent with the finding that kisspeptin detection in the hypophyseal portal system does not change during the time of the GnRH/LH surge [60]. Thus, a role for KNDy cells and the proposed kisspeptin/GnRH axo-axonal mechanism in the generation of the LH surge requires further investigation.

The lower level of NKB expression in kisspeptin neurons during the luteal phase of the estrous cycle suggests a negative regulation of the gene by progesterone at this time, but this requires confirmation by ablation/replacement studies. In terms of the role of NKB in the follicular phase of the cycle, it seems most likely that the higher level of expression supports increased KNDy cell activity to stimulate higher rates of pulsatile GnRH/LH secretion at this time. On the other hand, the lack of change in NKB expression in the OVX ewes, given a surge-inducing challenge with estradiol [35], supports our present observations that a change in NKB function is not associated with the positive feedback mechanism (see above). It seems most likely that dynorphin is an important modulator during the luteal phase, since this peptide mediates the inhibition of GnRH/LH pulse frequency by progesterone [17, 18, 61, 62]. It has also been suggested that dynorphin inhibits KNDy neurons in a coordinated autosynaptic network in the generation of GnRH pulses [21, 23, 63, 64]. In the present study, we did not see any change in LH pulse frequency following the central administration of dynorphin, but these experiments were performed in the luteal phase, when progesterone exerts a strong negative feedback on the secretion of GnRH. Consistent with this, studies in goats show that dynorphin treatment decreased the LH pulse frequency, but only in OVX animals [30]. Wakabayashi et al. [30] concluded that dynorphin acts upstream (similar to NKB) of the kisspeptin effect on GnRH neurons in order to inhibit kisspeptin neurons and their output to generate GnRH/LH pulses. Expression of the dynorphin receptor (the κ-opioid receptor) is seen in only 20% of kisspeptin neurons in mice [21], but further experiments are

required to clearly define the role of dynorphin in the regulation of KNDy cells in other species.

Our data show an inconsistency in the colocalization of kisspeptin and NKB neurons when comparing data from in situ hybridization and immunohistochemistry. While we have observed that virtually all NKB-ir neurons are also kisspeptin-ir in the ovine ARC, as shown previously [19], there was only 55% coexpression of ^{35}S-labeled *Tac3* mRNA neurons with DIG-labeled *Kiss1* mRNA neurons. While the latter may indicate a significant population of NKB neurons that do not coexpress kisspeptin, it is also possible that it may reflect the level of detection. Consistent with this, the number of identifiable NKB *(Tac3)* mRNA cells is greater than that of NKB-ir cells, which is similar to our previous data regarding kisspeptin mRNA versus protein [13]. We believe the sensitivity of the two labeling methods is the major cause of this inconsistency, with the radioactive detection method being far more sensitive than DIG-based labeling or immunolabeling. On the other hand, the inconsistency between in situ hybridization and immunohistochemistry profiles for NKB may not be surprising, since peptide-expressing neurons can often be detected with mRNA but not protein in the cell body, suggesting a rapid transportation of peptide to terminals in these neurons. In this regard, it will be interesting to see whether there are different populations of NKB neurons in the ARC and/or differences in the transportation of NKB and kisspeptin peptide.

In conclusion, we have shown that NKB *(Tac3)* gene expression in kisspeptin neurons is higher in the late follicular phase of the ovine estrous cycle than in the luteal phase. These data support the hypothesis that NKB operates within kisspeptin neurons in the mid-region of the ARC to stimulate the tonic pulsatile release of GnRH/LH. Further indication that NKB is important for the generation of GnRH pulses was gained by showing inhibition of pulsatile LH secretion by central infusion of an NK3R antagonist. Moreover, the antagonist had no effect on the onset or amplitude of an estradiol-induced LH surge, indicating that NKB signaling is not essential to the activational stage of estrogen-positive feedback.

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Disclosure Statement

The authors have nothing to disclose.

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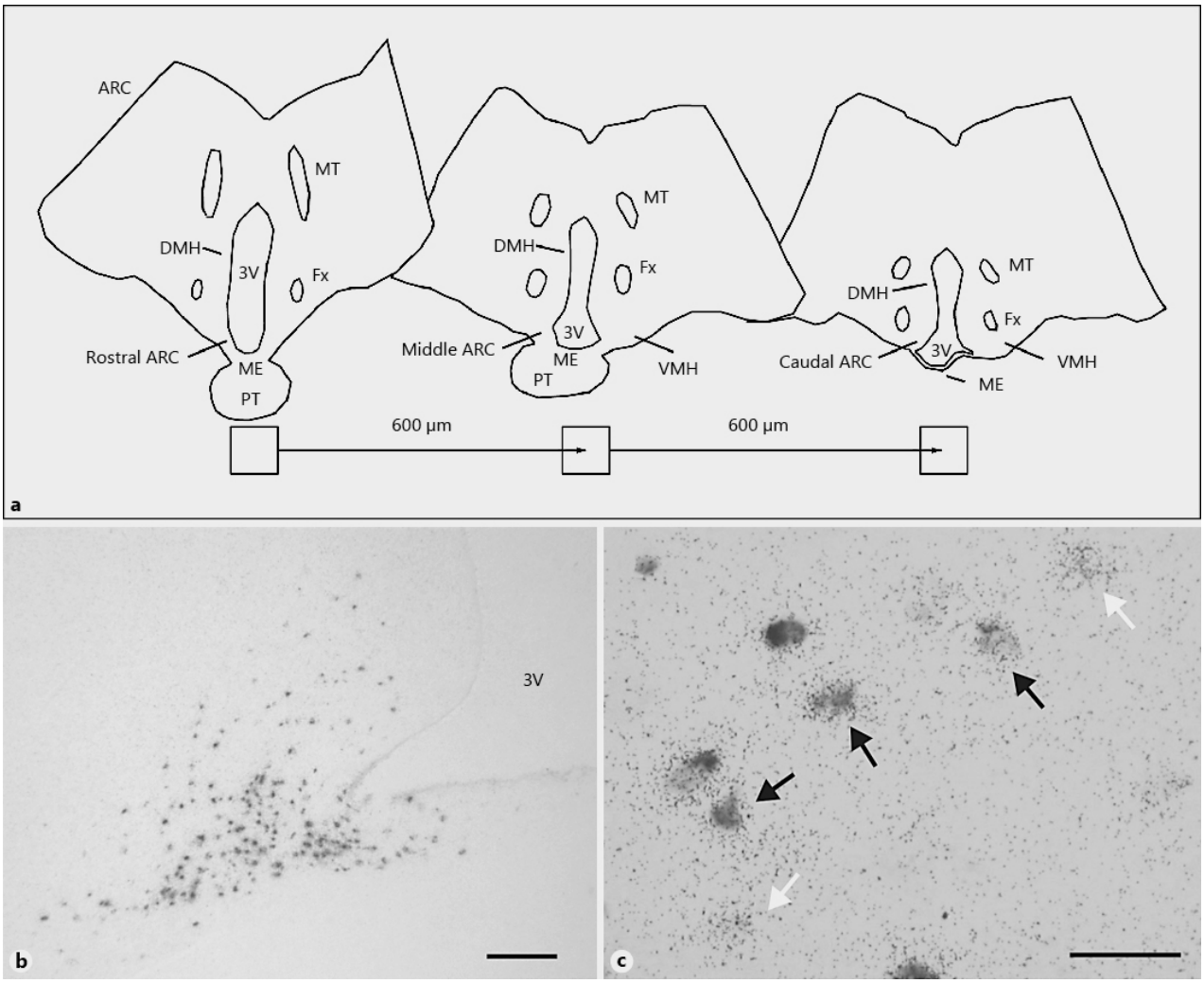
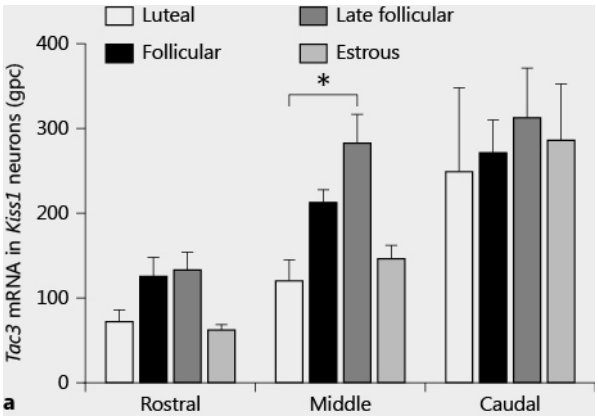


Fig. 1. Expression of Tac3 mRNA in Kiss1 mRNA--expressing neurons in the ARC. **a** Schematic drawing depicting representational coronal sections of the rostral, middle, and caudal ARC. Approximate distances between sections are indicated. DMH = Dorsomedial hypothalamus;; Fx = fornix;; MT = mammillothalamic tract;; PT = pars tuberalis;; VMH = ventromedial hypothalamus;; 3V = third ventricle. Modified from Smith et al. [13] with permission from the Endocrine Society. **b, c** Representative photomicrographs showing the distribution of Tac3 mRNA--containing neurons in the ARC as detected by 35S--labeled riboprobe (**b**) as well as colocalization with Kiss1 mRNA as detected by DIG--labeled riboprobe (**c**). The images are from late--follicular--phase ewes. Black arrows in c indicate the double--labeled neurons;; white arrows indicate Tac3 single--labeled neurons. Scale bars =100 µm (**b**) and 25 µm (**c**).


Luteal
Follicular
Late follicular
Estrous
400
300
200
100
0
*
Tac3 mRNA in Kiss1 neurons (gpc)
Rostral
Middle
Caudal
a

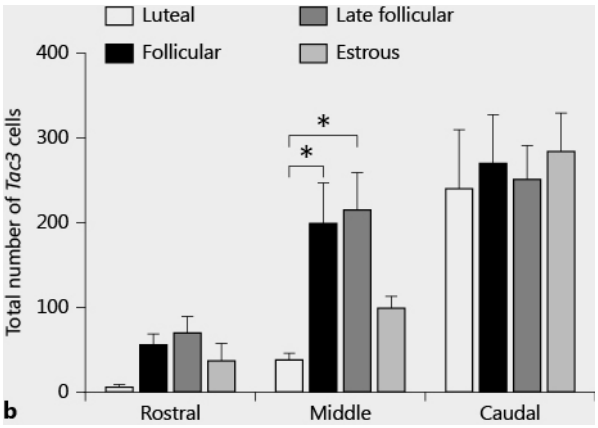
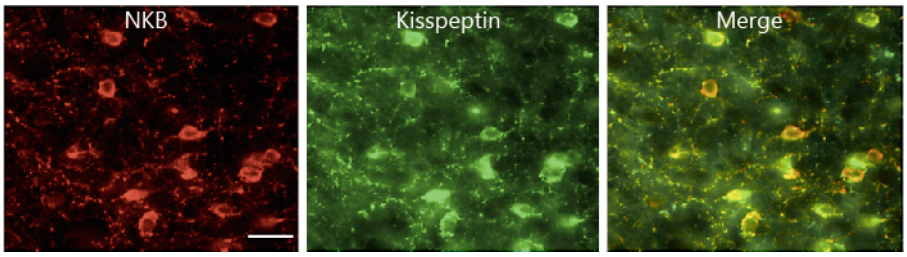


Fig. 2. Expression of Tac3 mRNA in Kiss1 mRNA--expressing neurons in the ARC across the ovine estrous cycle. **a** Per--cell--content expression of Tac3 in Kiss1 neurons in the rostral, middle, and caudal ARC across the estrous cycle (n = 3----6 per group). gpc = Silver grains per cell. **b** Total number of Tac3 mRNA--expressing neurons in the ARC across the estrous cycle (n = 3----6 per group). Values are means ± SEM. * $p < 0.05$.


NKB
Kisspeptin
Merge

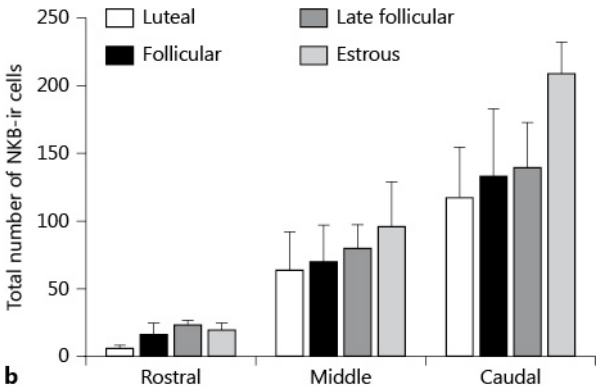
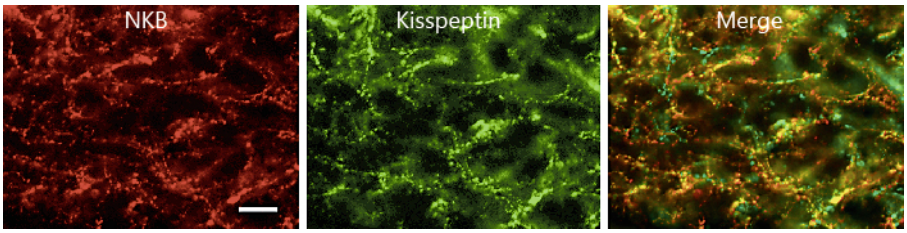
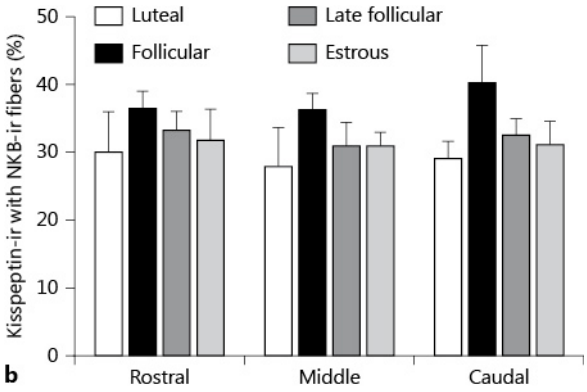
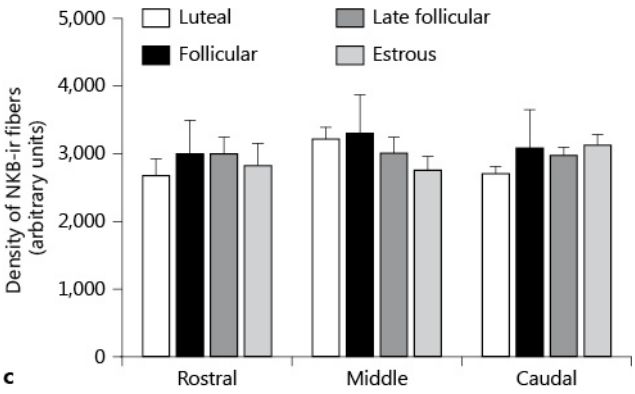


Fig. 3. Colocalization of NKB--ir and kisspeptin--ir neurons in the ARC as well as the number of NKB--ir neurons across the estrous cycle. **a** Representative photomicrographs showing the colocalization of NKB--ir and kisspeptin--ir staining in the ARC. Scale bar = 50 μm. **b** Number of NKB--ir neurons in the rostrocaudal sections across the estrous cycle (n = 3----6 per group). Values are means ± SEM.


NKB
Kisspeptin
Merge


Luteal
Late follicular
Follicular
Estrous
Kisspeptin-ir with NKB-ir fibers (%)
50
40
30
20
10
0
Rostral
Middle
Caudal
b


5,000
4,000
3,000
2,000
1,000
0
Density of NKB-ir fibers (arbitrary units)
Luteal
Follicular
Late follicular
Estrous
Rostral
Middle
Caudal
c

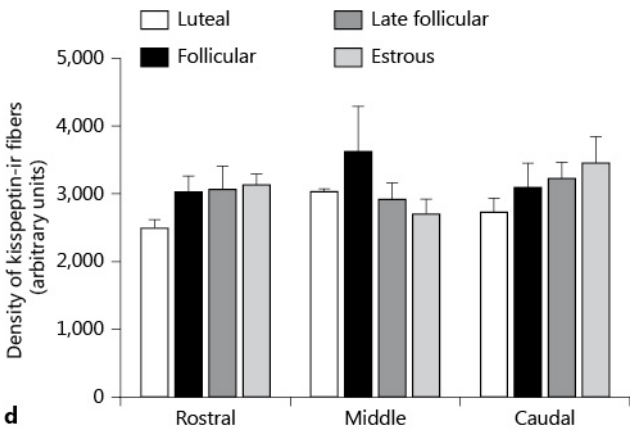


Fig. 4. Colocalization of NKB--ir and kisspeptin--ir terminals in the ME. **a** Representative photomicrographs showing NKB--ir and kisspeptin--ir terminals in the ME. Scale bar= 50 μm. **b** Colocalized NKB--ir and kisspeptin--ir fibers in the ME**. c** Density of NKB--ir fibers in rostrocaudal ME sections across the estrous cycle. **d** Density of kisspeptin--ir fibers in rostrocaudal ME sections across the estrous cycle (n = 3----6 per group). Values are means ± SEM.

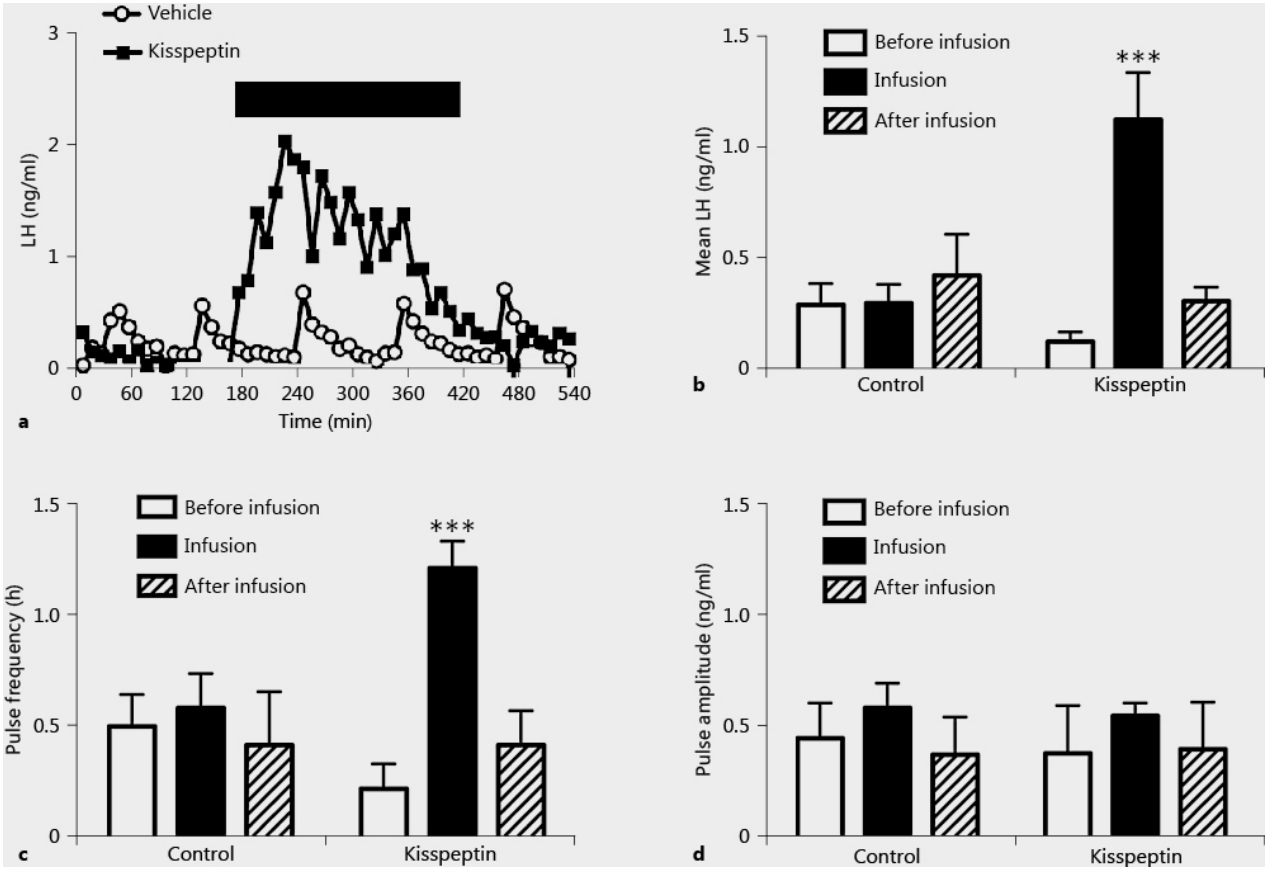


Fig. 5. Central infusion of kisspeptin stimulates the secretory pulses of LH in ewes. **a** **C**oncentrations of LH are shown in 2 representative animals treated with kisspeptin or aCSF (vehicle). The infusion period is represented by the closed bar. **b**-**d** Mean LH **(b),** LH pulse frequency **(c),** and pulse amplitude **(d) b**efore, during, and after infusion (n = 6 per group). Data are means ± SEM. *** $p < 0.001$ compared to before infusion.

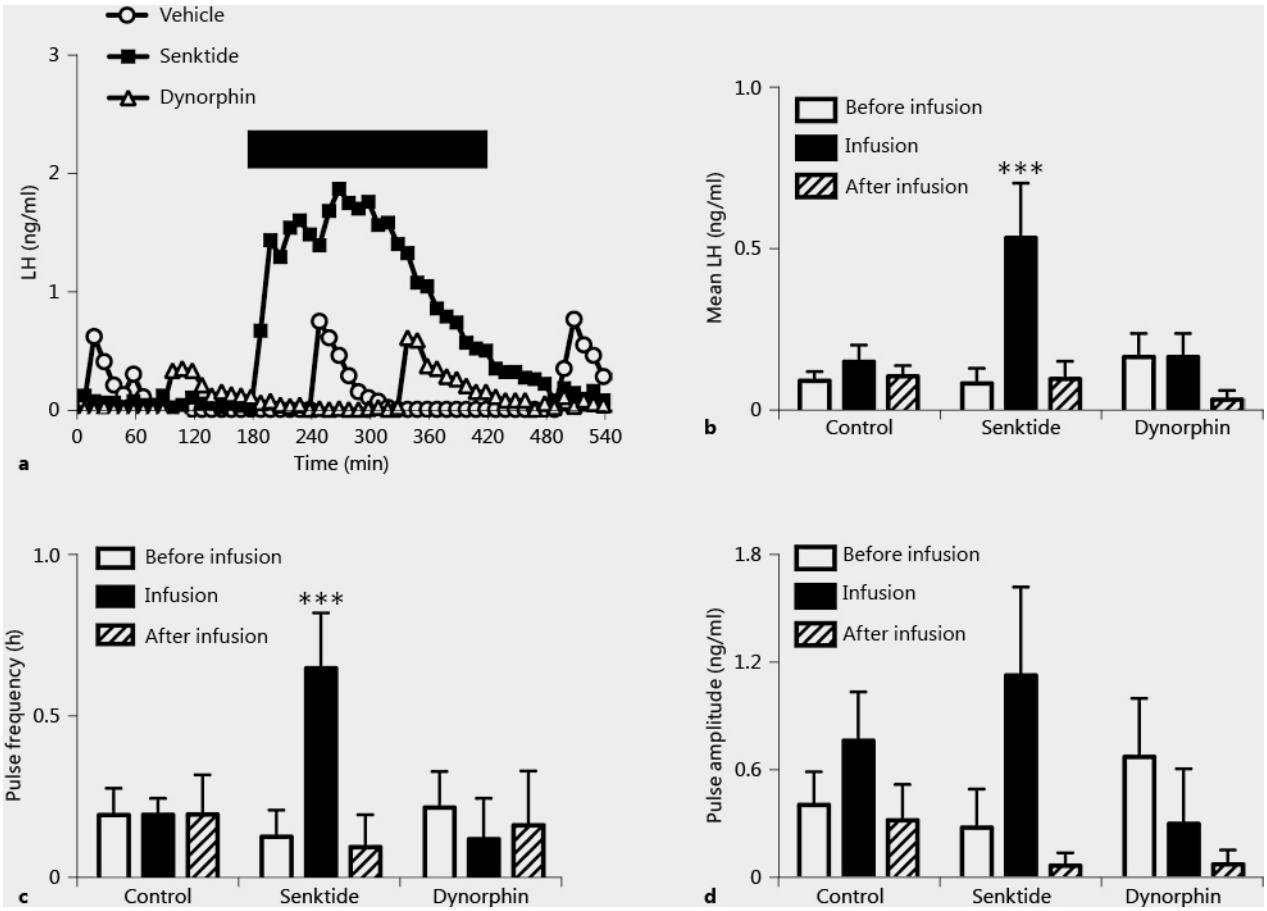


Fig. 6. Central infusion of senktide stimulates the secretory pulses of LH in ewes. **a** Concentrations of LH are shown in 3 representative animals treated with senktide, dynorphin, or aCSF (vehicle). The infusion period is represented by the closed bar. **b**--**d** Mean LH (**b**), LH pulse frequency (**c**), and pulse amplitude (**d)** before, during, and after infusion (n = 5 per group). Data are means ± SEM. *** $p < 0.001$ compared to before infusion.

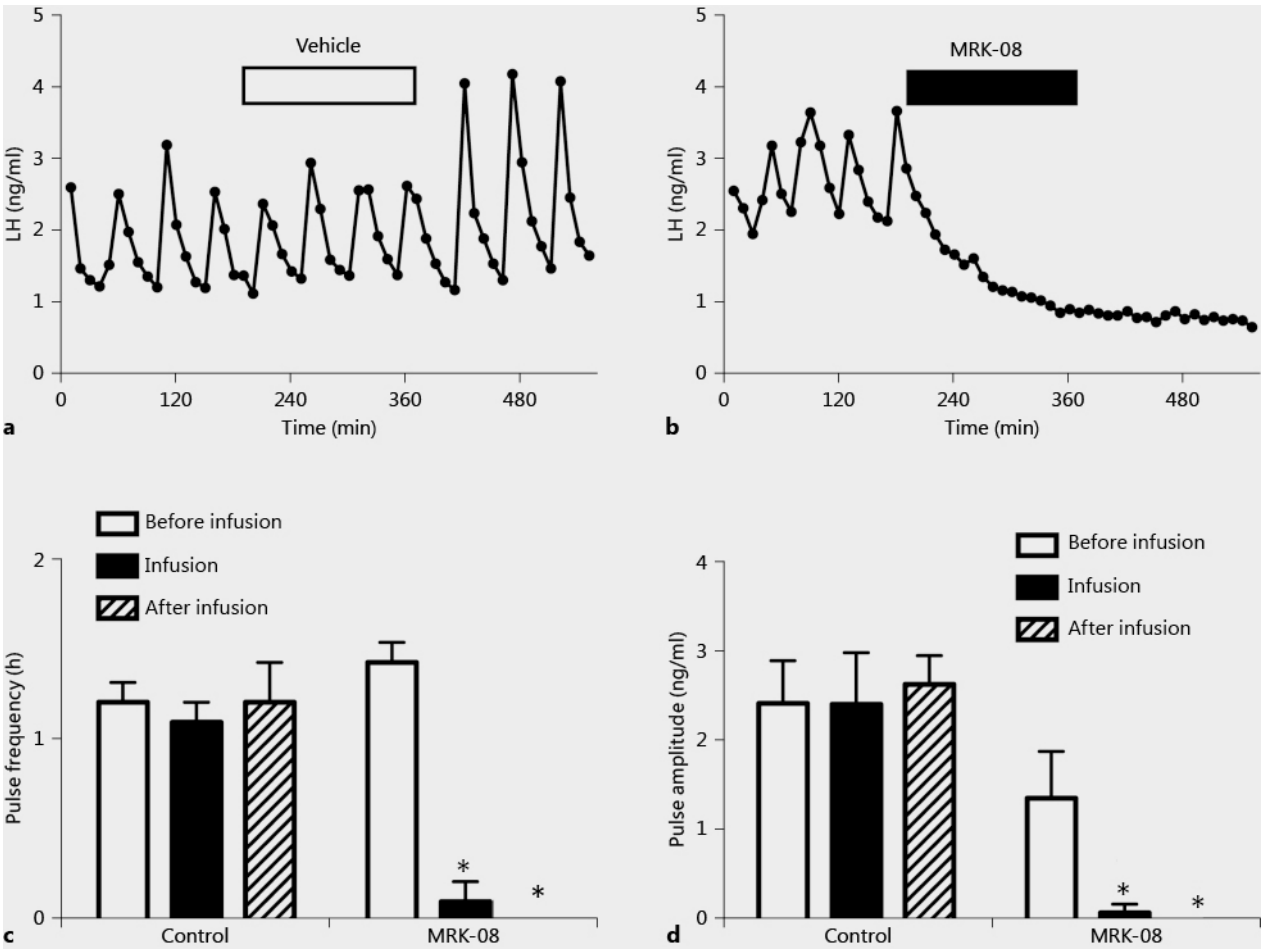


Fig. 7. Central infusion of the NK3R antagonist MRK--08 inhibits pulses of LH in OVX ewes. **a, b** Concentrations of LH are shown in representative animals treated with vehicle (**a,** open bar) or MRK--08 (**b,** closed bar). **c, d** LH pulse frequency (**c**) and pulse amplitude (**d**) during 3--hour time periods before, during, and after infusion (n = 3 per group). Data are means ± SEM. * $p < 0.05$ compared to before infusion.

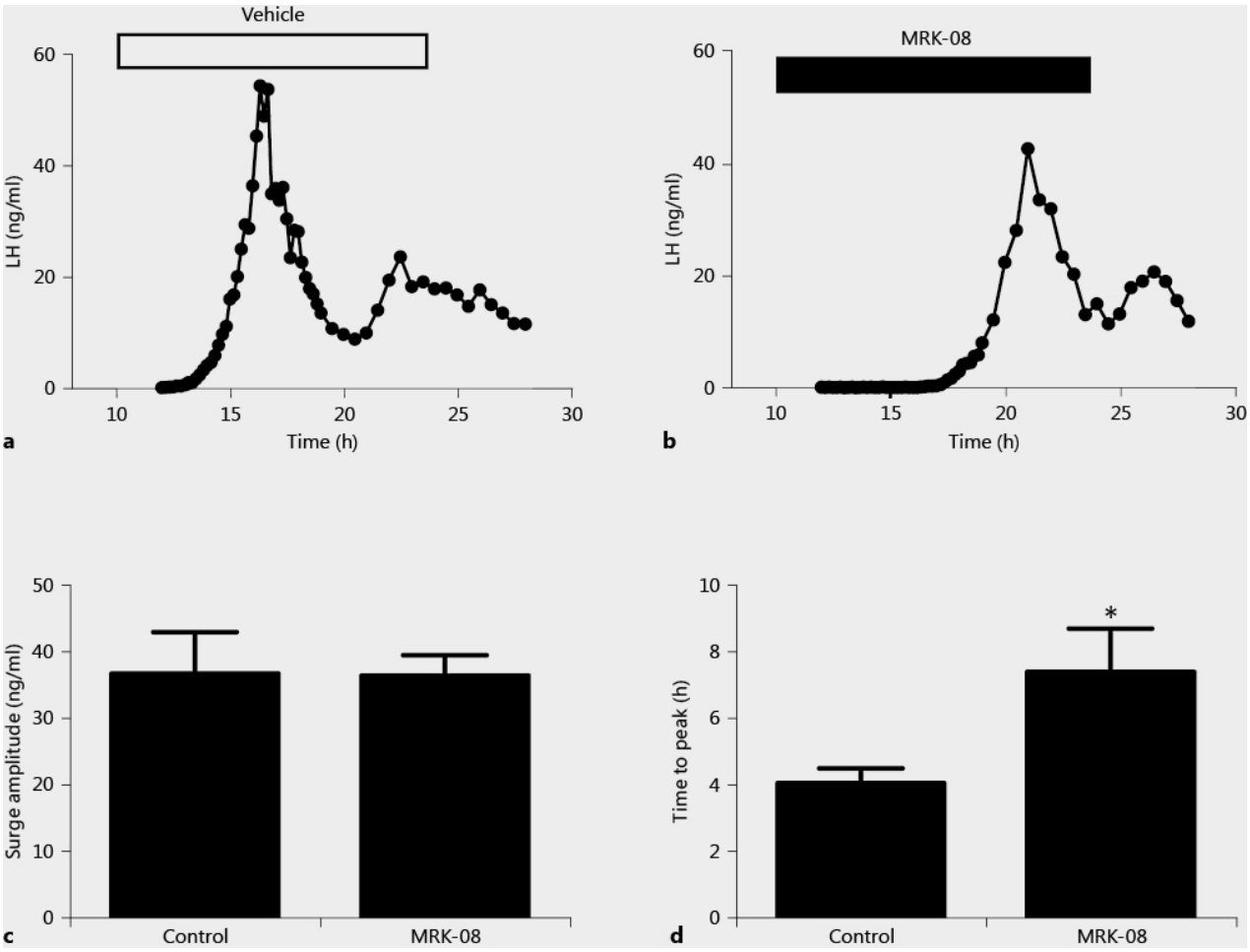


Fig. 8. Central infusion of the NK3R antagonist MRK--08 does not prevent an estradiol--induced LH surge. **a, b** Concentrations of LH are shown in representative animals treated with vehicle (**a,** open bar) or MRK--08 (**b,** closed bar;; n = 5 per group). The x--axis shows the time from estrogen treatment. **c** The LH surge amplitude was unchanged in ewes treated with MRK--08. **d** The time from LH surge onset to peak was longer in ewes treated with MRK--08. Data are means ± SEM. * $p < 0.05$.