## ORIGINAL ARTICLE

# Prenatal Testosterone Excess Decreases Neurokinin 3 Receptor Immunoreactivity within the Arcuate Nucleus KNDy Cell Population

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Prenatal exposure of the female ovine foetus to excess testosterone leads to neuroendocrine disruptions in adulthood, as demonstrated by defects in responsiveness with respect to the ability of gonadal steroids to regulate gonadotrophin-releasing hormone (GnRH) secretion. In the ewe, neurones of the arcuate nucleus (ARC), which co-expresses kisspeptin, neurokinin B (NKB) and dynorphin (termed KNDy cells), play a key role in steroid feedback control of GnRH and show altered peptide expression after prenatal testosterone treatment. KNDy cells also co-localise NKB receptors (NK3R), and it has been proposed that NKB may act as an autoregulatory transmitter in KNDy cells where it participates in the mechanisms underlying steroid negative-feedback. In addition, recent evidence suggests that NKB/NK3R signalling may be involved in the positive-feedback actions of oestradiol leading to the GnRH/luteinising hormone (LH) surge in the ewe. Thus, we hypothesise that decreased expression of NK3R in KNDy cells may be present in the brains of prenatal testosterone-treated animals, potentially contributing to reproductive defects. Using single- and dual-label immunohistochemistry we found NK3R-positive cells in diverse areas of the hypothalamus; however, after prenatal testosterone treatment, decreased numbers of NK3R immunoreactive (-IR) cells were seen only in the ARC. Moreover, dual-label confocal analyses revealed a significant decrease in the percentage of KNDy cells (using kisspeptin as a marker) that co-localised NK3R. To investigate how NKB ultimately affects GnRH secretion in the ewe, we examined GnRH neurones in the preoptic area (POA) and mediobasal hypothalamus (MBH) for the presence of NK3R. Although, consistent with earlier findings, we found no instances of NK3R co-localisation in GnRH neurones in either the POA or MBH; in addition, > 70% GnRH neurones in both areas were contacted by NK3R-IR presynaptic terminals suggesting that, in addition to its role at KNDy cell bodies, NKB may regulate GnRH neurones by presynaptic actions. In summary, the finding of decreased NK3R within KNDy cells in prenatal testosterone-treated sheep complements previous observations of decreased NKB and dynorphin in the same population, and may contribute to deficits in the feedback control of GnRH/LH secretion in this animal model.

**Key words:** neuroendocrine, developmental programming, reproduction, neurokinin B, kisspeptin, GnRH

A candidate afferent signalling system that has received much recent attention in the central control of gonadotrophin-releasing hormone (GnRH) secretion is that comprising the tachykinin neurokinin B (NKB) and its high affinity receptor neurokinin-3 (NK3R)

(1,2). Although NKB/NK3R signalling is implicated in diverse physiological functions (3), its importance in modulating gonadotrophin release was established when human genetic studies revealed that patients bearing inactivating mutations in the gene encoding NKB

(*TAC3*) or its receptor NK3R (as encoded by *TAC3R*) displayed hy-pogonadotrophic hypogonadism and infertility (4,5). Subsequently, a growing number of animal studies have established a close association between NKB/NK3R signalling and GnRH/luteinising hormone (LH) secretion in various species, including sheep (6), goat (7), primate (8) and rat (9). However, the precise neuronal pathway(s) via which NKB stimulates GnRH secretion are not yet fully determined. Although a subset of GnRH neurones in the rat have been shown to co-localise NK3R (10), similar studies in sheep (11) and mice (12) have failed to reveal NK3R in GnRH neurones, suggesting that the action of NKB upon GnRH secretion is likely exerted via inputs from other neurones, either directly or indirectly.

The neuroanatomical location of NK3R has been described previously in the ewe and includes NK3R-immunoreactive (-IR) cells in a variety of preoptic and hypothalamic nuclei, including the preoptic area (POA), retrochiasmatic area (RCh) and arcuate nucleus (ARC) (11). In the ARC, NKB is co-localised with two other neuropeptides, kisspeptin and dynorphin, in a population that is termed KNDy (kisspeptin, neurokinin B and dynorphin) cells (13). KNDy cells are present in the ARC of all species studied to date (14) and are considered to play a key role in the negative-feedback effects of oestradiol and progesterone upon GnRH (14). In addition, KNDy cells are considered to comprise a critical component of the circuitry responsible for the generation of GnRH/LH pulses (7,9,16). Accumulating evidence suggests that NKB acts as an auto-regulatory signal within the network of reciprocally interconnected KNDY cells, comprising a signal that is responsible for the initiation of each GnRH pulse (16).

In addition to its role in negative-feedback, in the sheep, NKB/NK3R signalling may also be important in the generation of the preovulatory GnRH/LH surge (13,17–19). Intracerebroventricular microinjections of senktide, a NK3R specific agonist (2), results in a surge-like elevation of LH during the follicular but not the luteal phase of the ovine oestrous cycle (6). Bilateral senktide microinjections into the RCh (6) and POA (20) are each able to produce a similar surge-like elevation of LH, suggesting that these two areas, each of which contain NK3R-positive cells, may participate in the control of the LH surge. In addition, there are several lines of evidence suggesting that KNDy cells may participate in the GnRH/LH surge in the ewe (18,19,21–24). For example, in the sheep (17), unlike rodents (25), oestradiol implants in the mediobasal hypothalamus, close to the vicinity of KNDy cells, are sufficient to induce a GnRH/LH surge. Thus, NKB/NK3R signalling could potentially play a role in both the negative- and positive-feedback effects of gonadal steroids in the sheep, acting at potential target sites that include the POA, RCh and KNDy cells of the ARC.

The responsiveness of the adult GnRH system to hormonal feedback controls is programmed during development by events that include foetal exposure to androgens (26,27). Although normal sexual differentiation depends on appropriate timing of exposure of foetuses to androgens, exposure to excess androgens in animal models can result in long-term deficits in reproductive functions at multiple levels, including the GnRH system (28). For example, exposure of female ovine foetuses to excess testosterone during days 30–90 of the 147-day gestation, leads to neuroendocrine defects in the responsiveness of the GnRH system to both negative and positive steroid feedback (29–32). KNDy neurones have been implicated as critical mediators of the detrimental effects of prenatal testosterone (33), and prenatal testosterone treatment results in dramatic alterations in KNDy peptides in the adult ARC, with NKB and dynorphin being markedly reduced but kisspeptin remaining unaltered. This peptide imbalance within a single neuronal population has been hypothesised to underlie some of the defects in responsiveness of the GnRH system to oestradiol and progesterone seen in adult female sheep exposed prenatally to excess testosterone (33).

Whether postsynaptic receptors for any of the KNDy peptides are similarly altered in prenatal testosterone-treated animals has not yet been examined and, given the evidence for participation of NKB/NK3R signalling in both pulsatile and surge modes of GnRH/LH secretion, we hypothesised that changes in NK3R expression in either the ARC or in other regions where it has been shown to alter LH secretion (e.g. RCh, POA) may be present in the brains of prenatal testosterone-treated female sheep. To test this hypothesis, we first compared the overall number of NK3R-IR cells in the ARC, RCh, POA and other hypothalamic nuclei between prenatal testosterone-treated and control animals. Second, we used dual-label immunofluorescence and confocal microscopy to determine whether NK3R might be specifically altered within the KNDy cell subpopulation of the ARC. Finally, because NK3R-IR is seen in fibre and terminals, as well as cell bodies, we explored the possibility that NKB might act presynaptically to influence GnRH secretion by determining whether NK3R-IR terminals in the POA and medial basal hypothalamus (MBH) are in direct synaptic contact with GnRH cell bodies in those regions. To control for the possible influence of differences in circulating steroids between the experimental and control groups, animals were ovariectomised prior to sacrifice and implanted with hormonal regimens designed to produce late follicular phase concentrations of oestradiol.

## Materials and methods

### Animal care and treatment

All procedures involving animals were approved by the University of Michigan Animal Care and Use Committee and are consistent with National Research Council's Guide for the Care and Use of Laboratory Animals. The experiments were conducted in 2-year-old control and prenatal testosterone-treated Suffolk ewes during the breeding season. Housing, breeding, lambing and maintenance took place at the Sheep Research Facility at the University of Michigan (Ann Arbor, MI, USA; 42°18′ north latitude) as described previously (34,35).

Pregnant ewes were administered i.m. injections of testosterone propionate (100 mg/injection catalog item T1875; Sigma-Aldrich, St Louis, MO, USA; n = 8) twice weekly, suspended in cottonseed oil (catalogue number C7767; Sigma-Aldrich) in the hind leg from days 30 to 90 of pregnancy (term = 147 days). The dose of testosterone propionate administered results in levels of testosterone in the female foetus comparable to those in foetal males (36). Control ewes received an equal volume of vehicle (2 ml cottonseed oil; n = 9) with the same regimen as testosterone. Lambs were born in March/April. After weaning, they were maintained outdoors under natural

photoperiods with a daily maintenance feeding and free access to water. During the first breeding season, progesterone profiles and ultrasonographic assessment of ovarian status was carried out (37). In addition, at approximately 19 months of age, all ewes were ovariectomised and evaluated for LH surge and sexual behaviour profiles (38). For the present study, and to normalise the hormonal milieu between all animals, a 1-cm long Silastic capsule (inner diameter 3.35 mm; outer diameter 4.65 mm; Dow Corning Corp., Midland, MI, USA) filled with 17β oestradiol (oestradiol; Sigma-Aldrich) and two controlled internal drug release (CIDR) progesterone implants (InterAG, Hamilton, Waikato, New Zealand) were inserted s.c. into each animal. CIDRS were removed 14 days later and, sequentially, all animals received additional four 3-cm long oestradiol implants (see above) to simulate ovarian steroid levels equivalent to a normal follicular phase, as well as to generate a GnRH/LH surge (41). Animals were euthanised approximately 20 h after insertion of the oestradiol implants and specifically during the late follicular phase (i.e. prior to the LH surge in both control or testosterone-treated animals) (38). The oestradiol implant insertion and euthanasia where staggered at hourly intervals to allow for the time needed to perfuse and extract the brain.

## Tissue collection and preparation

Ewes were injected i.v. twice at 10-min intervals with 25 000 U of heparin (catalogue number 402588B; Abraxiz Pharmaceutical Products, Schaumburg, IL, USA) and then deeply anaesthetised with i.v. sodium pentobarbital (2–3 g; catalogue number P3761; Sigma-Aldrich). Animals were rapidly decapitated, and the heads perfused via both internal carotids with 6 litres of 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.3) mixed with 0.1% sodium nitrite and administered with 10 U/ml heparin. After perfusion, the brain was removed and a tissue block containing the septal region, POA and hypothalamus dissected out. Blocks were incubated in 4% paraformaldehyde at 4 °C overnight for post-fixation and then transferred into 30% sucrose in 0.1 M PB for cryoprotection until infiltration took place. A sliding freezing microtome (SM 200R; Leica Biosystems, Walldorf, Germany) was used to section frozen blocks of tissue containing POA and hypothalamus into six series of coronal 45-μm slices. Free-floating sections were stored in cryoprotectant solution (30% ethylene glycol, 1% polyvinylpyrrolidone, 30% sucrose in sodium phosphate buffer) (42) at −20 °C until processed for immunohistochemistry. Within each experiment, tissue sections from all experimental groups were processed simultaneously as described below. All immunohistochemical procedures were carried out at room temperature under gentle agitation. Unless otherwise stated, tissue sections were washed with 0.1 M phosphate-buffered saline (PBS; pH 7.2) between steps. Antibodies were diluted with blocking solution, comprising 0.4% Triton X-100 (catalogue number BP151-500; Sigma-Aldrich) and 4% normal goat serum (NGS; catalogue number H005-000-121; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) in 0.1 M PBS.

## Experiment 1: Effects of prenatal testosterone-treatment on NK3R-IR in the POA and hypothalamus

### Single-label immunohistochemistry for NK3R

The distribution and quantification of NK3R-IR cells was determined in a series of every sixth section (270 μm apart). Free-floating sections were washed thoroughly in 0.1 M PBS for several hours to remove excess cryoprotectant, followed by a 10-min incubation with PBS containing 1% hydrogen peroxide (H_2O_2; catalogue number H325; Fisher Scientific, Pittsburgh, PA, USA) to eliminate endogenous peroxidase activity. Next, sections were incubated in blocking solution for 1 h followed by an overnight (17 h) incubation with polyclonal rabbit anti-NK3R (dilution 1 : 10 000; catalogue number NB300-102; Novus Biological, Littleton, CO, USA). After incubation with the primary antiserum, sections were incubated with biotinylated goat anti-rabbit immunoglobulin G (dilution 1 : 500; catalogue number BA-1000; Vector Laboratories, Burlingame, CA, USA) for 1 h followed by incubation with ABC reagent (dilution 1 : 500 diluted in 0.1 M PBS; avidin and biotinylated horseradish peroxidase macromolecular complex, catalogue number PK-6100; Vector Laboratories) for 1 h. NK3R labelling was visualised using 3,3′-diaminobenzidine tetrahydrochloride (0.2 mg/ml) (catalogue number D5905; Sigma-Aldrich) with 0.00004% hydrogen peroxide in PB as substrate. Finally, they were mounted onto Superfrost/Plus Microscope Slides (Fisher Scientific), air-dried and coverslipped with DPX Mountant. Omission of NK3R antibody from the immunohistochemical protocol resulted in complete absence of staining. Furthermore, preabsorption controls with purified antigen have been performed and described previously (see below) (11).

## Experiment 2: Effects of prenatal testosterone-treatment on NK3R-IR within the POA kisspeptin and ARC KNDy cell population

### Dual-label immunofluorescent detection of NK3R and kisspeptin

To determine whether changes in NK3R-IR occurred specifically within the POA kisspeptin or ARC KNDy cell population, an alternate series of every sixth section (270 μm apart) containing the POA or ARC was processed for dual-label immunofluorescence and confocal microscopic analysis. A modification of the protocol reported by Hunyady *et al.* (43) was carried out to eliminate possible cross-linking between kisspeptin and NK3R antibodies (both raised in rabbits) and false co-localisation between antigens. Initially, free-floating tissue sections were washed several hours in PBS for cryoprotectant removal. Subsequently, they were incubated in PBS containing 1% H_2O_2 for 10 min followed by a 1-h incubation in blocking solution (with 20% NGS). Next, rabbit polyclonal anti-NK3R (dilution 1 : 10 000 for 17 h) was applied. Sections were then incubated sequentially in biotinylated goat anti-rabbit (dilution 1 : 500 for 1 h) and ABC-elite solution (dilution 1 : 500 diluted in 0.1 M PBS for 1 h). After amplification with TSA Biotin system Biotinyl Tyramide agent (dilution 1 : 250 diluted in 0.1 M PBS with 3% H_2O_2; catalogue number NEL700A001KT; PerkinElmer Life Sciences, Waltham, MA, USA), NK3R was visualised with Alexa 488 conjugated streptavidin (dilution 1 : 100 diluted in 0.1 M PBS for 30 min; catalogue number S-32354; Invitrogen/Molecular Probes, Eugene, OR, USA). Sections were then processed for detection of kisspeptin. First, they were incubated for 17 h with primary antibody rabbit anti-kisspeptin (a gift from A. Caraty, Universite Tours, Nouzilly, France; lot number 564) at a dilution of 1 : 2000 (for POA sections) or 1 : 10 000 (for ARC sections) and visualised with goat anti-rabbit Alexa 555 (dilution 1 : 100 in 0.1 M PBS for 30 min; catalogue number A-21428; Invitrogen/Molecular Probes). Finally, sections were mounted on glass slides, dried and coverslipped with mount medium gelvatol. Control sections for the dual immunofluorescent procedure included the omission of each of the primary antibodies from the immunostaining protocol, which resulted in a complete absence of staining for the corresponding antigen. In addition, pre-absorption controls have been performed for each of the antibodies in previous studies (11); in each case, pre-incubation of the diluted antiserum with nanomolar concentrations of purified antigen was shown to be sufficient to eliminate all specific staining in ewe hypothalamic sections. Finally, the kisspeptin antibody used has been shown to be specific for kisspeptin cells of the ovine brain and not to cross-react with other RFamide peptides (44).

## Experiment 3: Identification of pre-synaptic NK3R terminals onto GnRH neurones in the POA and MBH

### Triple-label immunofluorescent detection of GnRH, NK3R and synaptophysin

A series of every 12th section (540 μm apart) through the POA and MBH were used for GnRH/NK3R/synaptophysin triple labelling. Similar to the protocols described above, free-floating sections were washed in 0.1 M PBS for several hours to remove cryoprotectant. Next, they were incubated in 1% $H_2O_2$ diluted in PBS for 10 min, followed by a 1-h incubation in blocking solution (with 20% NGS). Subsequently, sections were incubated sequentially in rabbit polyclonal anti-NK3R, biotinylated goat anti-rabbit, ABC-elite solution and TSA Biotin system Biotinyl Tyramide agent, as described above. NK3R was visualised with Alexa 488 conjugated streptavidin (dilution 1 : 100 in 0.1 M PBS for 30 min). The second primary antibody, rabbit anti-GnRH (dilution 1 : 1000; LR-5, a gift from R. Benoit, Montréal General Hospital, Montréal, Canada), was visualised using indirect detection with goat anti-rabbit Alexa 555 (dilution 1 : 100 in 0.1 M PBS; catalogue number S-32354; Invitrogen/Molecular Probes). During the GnRH antibody incubation period, mouse anti-synaptophysin (dilution 1 : 200; catalogue number S5768; Sigma-Aldrich) was also co-incubated and visualised with donkey anti-mouse Cy5 (dilution 1 : 100 in 0.1 M PBS for 30 min; Catalogue number 715175151; Jackson ImmunoResearch Laboratories). Controls omitting one, two or all three primary antisera from the protocol completely eliminated all specific staining for the corresponding antigen(s).

## Data analysis

For single-label NK3R, the distribution of IR cells and fibres was examined in sections through the POA and hypothalamus of each ewe. Three representative sections of the rostral, middle and caudal divisions of the ARC, RCh, ventral portion premammillary nucleus, POA, lateral hypothalamic area and paraventricular nucleus were quantitatively analysed per animal in each group. Each nucleus was determined by its cytoarchitectonic boundaries and all cells within those boundaries were quantified. Areas chosen for analysis were based on the regional distribution of NK3R-IR cells previously described in the ewe (11). The ARC, which contains prominent NK3R-IR, was divided into three rostral–caudal divisions for more detailed analysis in the present study as described previously (11,45). Our preliminary observations revealed that the rostral ARC contained very few NK3R-IR cells and fibres compared to the middle and the caudal ARC. Moreover, given that a large majority of KNDy cells are found in the middle and caudal divisions (45,46), we selected these subregions for detailed comparison between control and prenatal testosterone-treated animals.

For single-label analyses (Experiment 1), NK3R-IR cells were examined and quantified with a DMRD microscope (Leica Microsystems GmbH, Wetzlar, Germany) and identified by the presence of dense reaction product that labelled their somas and dendrites. Images were captured using a digital camera (Magnafire; Optronics, Goleta, CA, USA) attached to the microscope and imported into PHOTOSHOP, version 7.0 (Adobe Systems, San Jose, CA, USA). Photomicrographs were not altered in any way, except for minor adjustments of brightness and contrast.

Sections processed for dual and triple immunofluorescence were analysed using a Zeiss LSM-510 laser-scanning confocal microscope system (Zeiss, Thronwood, NY, USA). Alexa 488 fluorescence was visualised and imaged with a 505–530-nm emission filter and argon laser, whereas Alexa 555 and CY5 fluorescence with a 560-nm and 680-nm emission filter and a HeNe laser. Confocal Z-stacks of optical sections (1 μm at × 63 magnification) were captured through NK3R, kisspeptin and GnRH-IR neurones. Three Z-stacks from the middle and caudal ARC of each animal were used for

analysis of NK3R/kisspeptin co-localisation. A total of 700 kisspeptin-IR cells from the middle ARC and 692 cells from the caudal ARC (between 38 and 42 kisspeptin-IR cells per treatment group and ARC subdivision) were analysed. For examination of possible co-localisation in the POA, a total of 42 kisspeptin-IR cells from three control animals (between 12 and 16 kisspeptin-IR cells per animal) were analysed.

For analysis of GnRH/NK3R/synaptophysin material, six to 10 Z-stacks were captured from the POA and ARC to obtain sufficient number of GnRH-IR neurones for analysis. Putative contacts between NK3R/synaptophysin-positive terminals and GnRH-IR somas were defined as a direct apposition without any intervening (black) pixels. A total 49 POA GnRH neurones and 34 MBH GnRH neurones were analysed from five random control animals (between seven and 12 POA and six or seven MBH GnRH neurones per animal). First, the somal perimetre was calculated by tracing the neurone. Subsequently, in each Z-stack of 1-μm optical section, the number of NK3R-positive terminals in direct contact with the GnRH neurone was determined. The percentage of GnRH neurones in the POA and MBH having one or more NK3R-positive contacts was calculated, as was the mean number of NK3R-positive contacts onto GnRH somas per animal, and the mean number of contacts per 10 μm of GnRH somal perimeter.

## Statistical analysis

All data are expressed as the mean ± SEM. Statistical comparisons between control and prenatal testosterone-treated ewes (Experiments 1 and 2) and between brain regions (Experiment 3) were made using Student's t-test. All statistics were performed using SIGMASTAT windows (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

## Results

### Experiment 1: Effects of prenatal testosterone-treatment on NK3R-IR cell numbers in the POA and hypothalamus

NK3R-IR cells were present in a number of areas of the hypothalamus in addition to the ARC (Fig. 1). The most prominent and dense populations of NK3R-IR neurones, other than the ARC, were observed (in descending order of overall cell number) in the hypothalamic paraventricular nucleus, lateral hypothalamic area, ventral premammillary nucleus, RCh and POA. In the ARC, where KNDY cells reside, we confirmed a large number of NK3R-IR cells, specifically in the middle and caudal divisions of this nucleus (Fig. 1).

Quantitative cell counts revealed that the mean number of NK3R-IR cells observed in the ARC of control ewes was significantly greater than that of prenatal testosterone-treated animals in both the middle (control: 53.8 ± 2.9 versus prenatal testosterone: 41.6 ± 2.8; P = 0.009) and caudal portions (control: 42.7 ± 4.0 versus prenatal testosterone: 30.0 ± 2.5; P = 0.019; Fig. 2) of this nucleus. No significant differences in NK3R-IR cell number between control and prenatal testosterone-treated animals were observed in any of the other nuclei or areas analysed (Fig. 2).

### Experiment 2: Effect of prenatal testosterine-treatment on NK3R-IR co-localisation within the ARC KNDy cell population

To determine whether changes in NK3R-IR cell number observed in the ARC, reflect a change in NK3R specifically in the KNDy cell
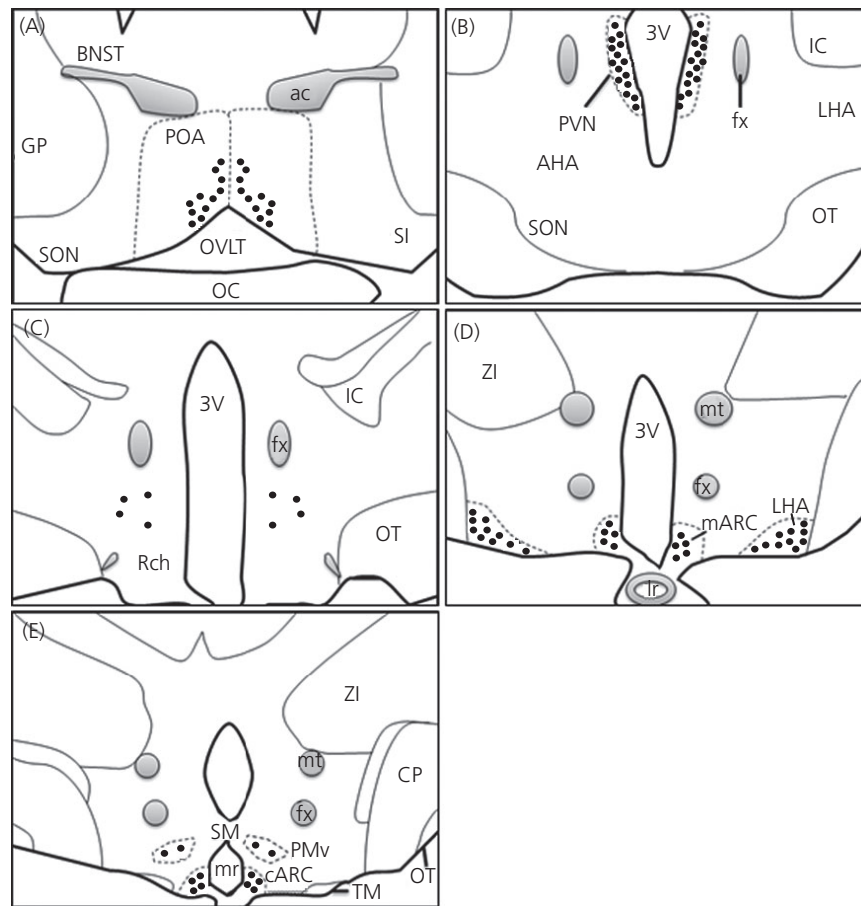
**Fig. 1.** Schematic drawings of coronal sections through the ovine preoptic area (POA) and hypothalamus, depicting the distribution of neurokinin-3 receptor-immunoreactive (NK3R-IR) cells. Each solid circle represents approximately 10 NK3R-IR cells. (A) BNST, bed nucleus of stria terminalis; GP, globus pallidus; ac, anterior commissure; SON, superior optic nucleus; OVLT, organum vasculosum of lamina terminalis; SI, substantia innominata; OC, optic chiasm; (B) fx, fornix; PVN, paraventricular nucleus; 3V, third ventricle; IC, internal capsule; AHA, anterior hypothalamic area; OT, optic tract; LHA, lateral hypothalamic area; (C) RCh, retrochiasmatic area; (D) ZI, zona incerta; mt, mammillary tract; mARC, middle arcuate; (E) CP, cerebral peduncle; PMv, premammillary ventricle; cARC, caudal arcuate; mr, mammillary recess.

population, we analysed sections processed for dual-immunofluorescent localisation of NK3R and kisspeptin (Kiss). Prenatal testosterone-treated animals showed a decrease in the number of dual-labelled NK3R/Kiss cells (control: $19.4 \pm 1.7$ versus prenatal testosterone: $14.4 \pm 1.2$; $P = 0.049$; Fig. 3G), as well as the total number (single-labelled + dual-labelled) of ARC NK3R-IR cells (control: $25.9 \pm 2.1$ versus prenatal testosterone: $20.7 \pm 1.7$; $P = 0.021$; Fig. 3G). As in previous studies (33), we saw no difference between control and prenatal testosterone animals in the total number of Kiss cells (Fig. 3G) and, consistent with the decrease in number of dual NK3R/Kiss cells, the number of single-labelled Kiss cells was significantly higher in prenatal testosterone ewes (control: $21.5 \pm 1.6$ versus prenatal testosterone: $26.7 \pm 1.4$; $P = 0.038$; Fig. 3G).

We used the numbers of dual-labelled and total cells in individual animals to calculate the percentage of ARC Kiss-IR cells co-localising NK3R and, conversely, the percentage of NK3R-IR neurones co-localising Kiss. The mean percentage of Kiss-IR neurones co-localising NK3R was significantly decreased in prenatal

testosterone animals compared to controls (control: $47.1 \pm 3.0\%$ versus prenatal testosterone: $34.7 \pm 2.4\%$; $P = 0.005$; Fig. 3H). By contrast, there was no significant difference between control and prenatal testosterone-treated animals in the percentage of NK3R-IR neurones co-localising Kiss (Fig. 3H).

Because NK3R-IR cells are present in the POA (Figs 1 and 2), we also examined kisspeptin cells in the ovine POA for co-localisation of NK3R. However, the kisspeptin/NK3R co-localisation in the POA was infrequent and variable (mean $\pm$ SEM: $5.3 \pm 5.3\%$) such that further comparison with prenatal testosterone-treated animals was not pursued.

## Experiment 3: Co-localisation of NK3R-IR in presynaptic terminals contacting GnRH neurones

In addition to detecting NK3R-IR in cell bodies (Experiment 1), we also noted NK3R localisation in fibres and terminals throughout a number of hypothalamic regions, including the POA and MBH.
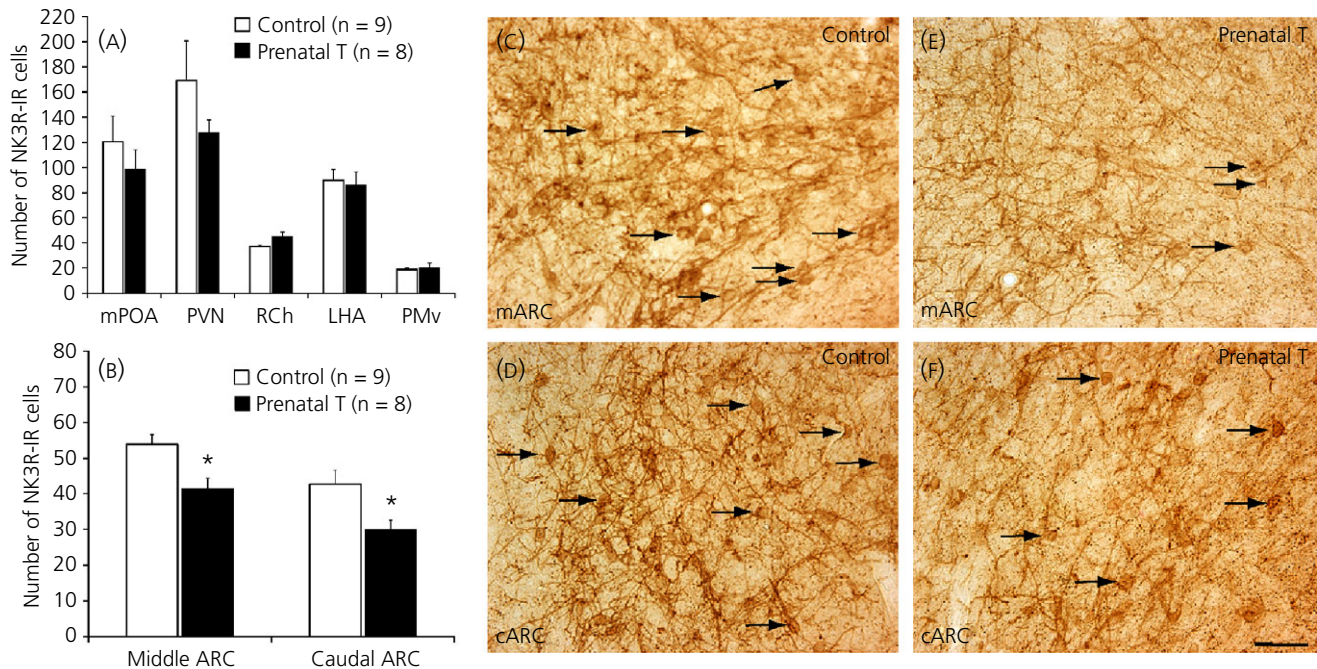
**Fig. 2.** (A) Mean ± SEM number of neurokinin-3 receptor-immunoreactive (NK3R-IR) cells/hemisection in the preoptic area (POA), paraventricular nucleus (PVN), retrochiasmatic area (RCh), lateral hypothalamic area (LHA) and premammillary ventricle (PMv) of control (n = 9) and prenatal testosterone (T)-treated (n = 8) groups. There were no statistically significant differences between control and prenatal testosterone-treated ewes in these areas. (B) Mean ± SEM number of NK3R immunoreactive cells/hemisection in the middle and caudal arcuate nucleus (ARC) from control (n = 9) and prenatal testosterone-treated (n = 8) groups. *Statistically significant differences within each subdivision compared to controls (P < 0.05). (C–F) Representative images showing examples of NK3R-IR cells (arrows) in the ARC of control (C, middle ARC; D, caudal ARC) and prenatal testosterone-treated ewes (E; middle ARC; F, caudal ARC). Scale bar = 50 μm.

Consequently, we processed sections for triple-label detection of NK3R, GnRH and synaptophysin to determine whether any of these NK3R-positive terminals were directly presynaptic to GnRH cell bodies in either the POA or MBH. Examination of triple-labelled sections showed that, from a total of 83 GnRH cells analysed (49 in the POA; 34 in the MBH), none contained NK3R, confirming our earlier results showing the lack of co-localisation of NK3R in ovine GnRH cells (11). However, NK3R-positive fibres were observed adjacent to, and intermixed with, GnRH cells and dendrites in both the POA and MBH. We found that > 70% of GnRH neurones examined were contacted by one or more NK3R-IR presynaptic bouton (defined by the co-localisation of synaptophysin; Fig. 4 and Table 1). Neither the percentage of GnRH cells receiving inputs, nor the mean number of inputs varied regionally between the POA and MBH (Table 1). Similarly, the mean number of contacts per 10-μm GnRH somal perimeter did not differ between the POA and MBH (Table 1).

## Discussion

Our results indicate that prenatal testosterone-treated ewes showed significantly diminished numbers of NK3R-IR neurones in the ARC compared to control animals. Furthermore, the decrease in NK3R was primarily a result of changes within the KNDy cell population and not in other ARC cells because the number of single-labelled

NK3R-IR cells (i.e. NK3R cells outside the KNDy cell population) in this region showed no difference between control and prenatal testosterone animals. The reduced number of NK3R-IR cells observed in prenatal testosterone female sheep parallels the decrease in numbers of NKB cells previously observed in this animal model (33) and suggests that the combined decrease in both ligand and receptor may contribute to defects in the control of GnRH/LH secretion.

Two possible functional consequences may be envisaged. One rests upon the proposed role of NKB/NK3R signalling in the generation of GnRH pulses (13). In the current model of GnRH pulse generation in ruminants (47), NKB serves as a 'start signal' that is responsible for initiation of each GnRH pulse and, by way of reciprocal connections, activates other NK3R-containing KNDy cells and ultimately GnRH neurones. Conceivably, decreased NKB and NK3R would lead to a diminished ability to initiate GnRH pulses and hence a decrease in GnRH/LH pulse frequency. However, prenatal testosterone animals show the opposite: an increase in LH pulse frequency in gonadal-intact ewes during anoestrus and the luteal phase of the oestrous cycle as a result of decreased responsiveness to the negative-feedback influence of oestradiol and progesterone, respectively (31,33,40). However, in addition to NKB, dynorphin peptide expression is also reduced in the KNDy cells of prenatal testosterone sheep (33). Evidence in ruminants supports the role of dynorphin in KNDy cells as a 'stop' signal, terminating each GnRH/LH pulse (16). Hence, it is possible that reductions in dynorphin
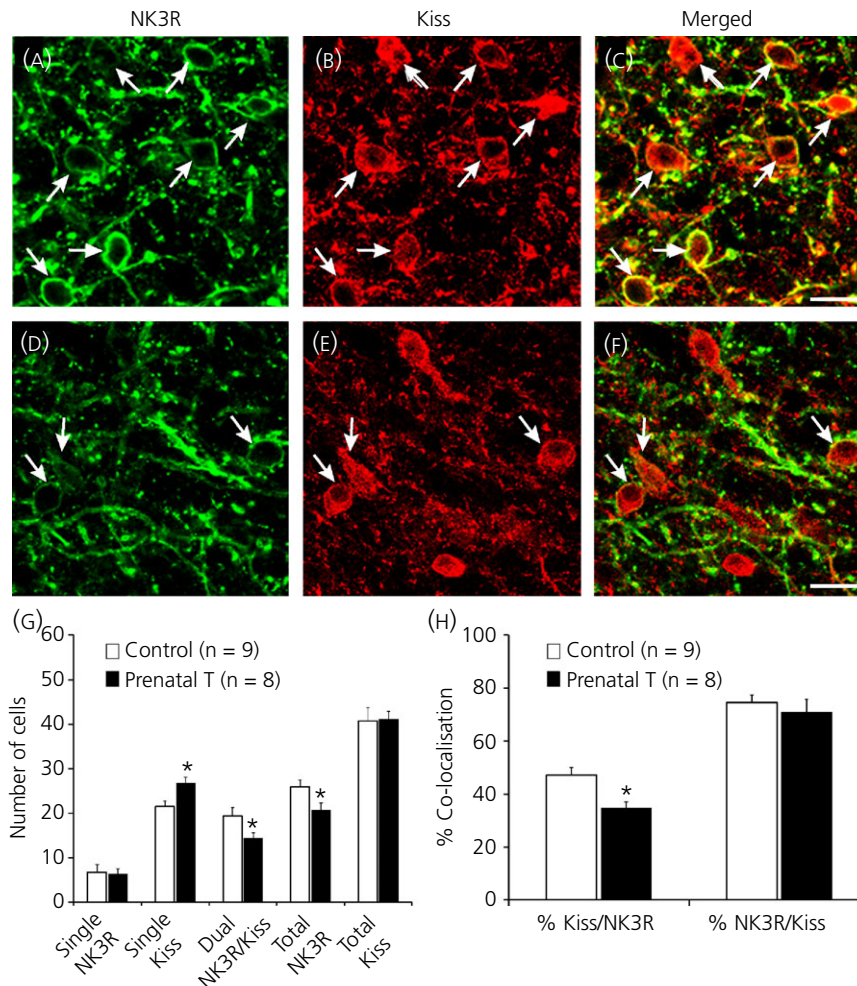
**Fig. 3.** (A–F) Confocal images (1-μm optical sections) showing dual-label immunofluorescent detection of neurokinin-3 receptor-immunoreactive (NK3R-IR) and kisspeptin-IR in the middle arcuate nucleus (ARC) of control (A–C) and prenatal testosterone (T)-treated ewes (D–F). Arrows indicate examples of dual-labelled neurones. Scale bar = 20 μm (× 63). (G) Mean ± SEM number of single-labelled NK3R, single-labelled kisspeptin (Kiss), dual-labelled NK3R and kisspeptin (NK3R/Kiss), and total kisspeptin and NK3R-IR neurones in the ARC of control (n = 9) and prenatal testosterone-treated (n = 8) ewes. (H) Mean ± SEM percentage of kisspeptin cells co-localising NK3R (%Kiss/NK3R; left) and percentage of NK3R cells co-localising kisspeptin (%NK3R/Kiss; right) in the ARC of control (n = 9) and prenatal testosterone-treated (n = 8) groups. *Statistically significant difference compared to controls (P < 0.05).

signalling compensate for that of NKB and NK3R, rendering the KNDy network less responsive to the negative-feedback influence of oestradiol and progesterone in prenatal testosterone animals. Nonetheless, although KNDy cells are known to be potential targets for direct actions of oestradiol and progesterone (13), we do not know whether these gonadal hormones inhibit GnRH/LH pulse frequency by acting directly on KNDy cells or indirectly via afferents from other cells. Evidence from KNDy cell-ablated rats suggests that, although KNDy cells participate in the negative-feedback influence of oestradiol on LH, other non-KNDy cells and pathways may also play a role (48). Similarly, despite the importance of NKB signalling on kisspeptin and hence GnRH stimulation, recent reports indicate that prenatal dihydrotestosterone treatment of peripubertal rats leads to elevated LH responses to i.c.v. kisspeptin administration (49). This, once again, is in accordance with decreased dynorphin expression rather than a reduction in NKB/NK3R signalling.

Another possibility is that decreased NKB/NK3R in prenatal testosterone sheep is related to deficits in the amplitude of the LH surge as seen in these animals (28,39). KNDy cells in the sheep express Fos, a marker of neuronal activation, during the preovulatory LH surge (19,24,18); i.c.v. injections of senktide, which elicit a surge-like elevation in LH, also induce Fos in ARC KNDy cells (20). Although kisspeptin mRNA and peptide expression in KNDy cells is increased during the late follicular phase in the ewe (21,22), an oestradiol stimulus that induced an LH surge was unable to increase in mRNA levels for NKB in the ARC (46). In addition, although NKB agonist injections locally into the POA and RCh, such as i.c.v. injections, result in a prolonged surge-like elevation of LH (see below), senktide injections in the ARC cause only a modest increase in LH (20) consistent with the role of NK3R in pulse generation in this region. Nonetheless, the possibility that NK3R signalling in KNDy cells plays a role in the generation of the LH surge in the ewe needs
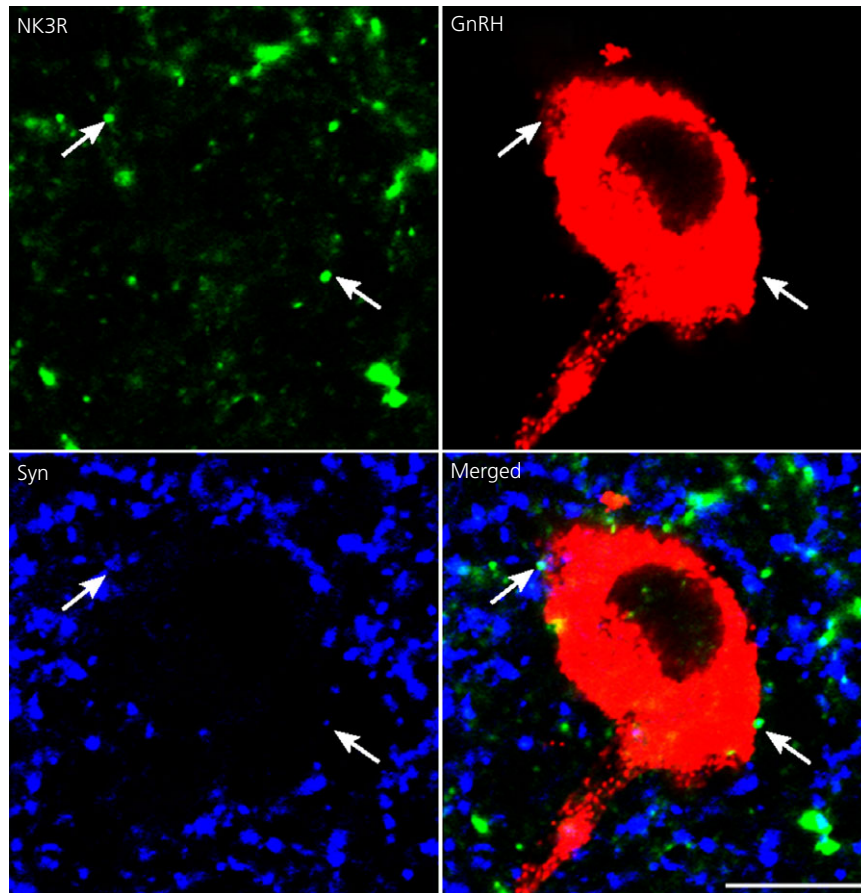
**Fig. 4.** Triple-label detection of neurokinin-3 receptor (NK3R), gonadotrophin-releasing hormone (GnRH) and synaptophysin (Syn) in a 1-μm confocal optical section, demonstrating the presence of presynaptic NK3R-immunoreactive terminals in contact with GnRH neurones in the mediobasal hypothalamus. Scale bar = 10 μm.

**Table 1.** Presynaptic Neurokinin-3 Receptor (NK3R) Terminals in Contact with Gonadotrophin-Releasing Hormone (GnRH) Somas: Mean ± SEM Percentage of GnRH Neurones Receiving One or More NK3R-Immunoreactive (-IR) Contact, Mean Number of NK3R-IR Contacts per GnRH Soma, and Mean Number of NK3R-IR Contacts per 10 μm Cell Surface, for GnRH Cells in the Pre-Optic Area (POA) and Mediobasal Hypothalamus (MBH) of Control Ewes (n = 5).

| GnRH cell bodies | POA | MBH | P |
|---|---|---|---|
| Percentage of somas receiving one or more NK3R contacts | 71.3 ± 8.1 | 79.1 ± 7.6 | 0.508 |
| Total number of NK3R contacts/soma/animal | 3.0 ± 0.6 | 4.8 ± 1.0 | 0.158 |
| Mean number of NK3R contacts/10-μm somal perimeter | 0.5 ± 0.2 | 0.8 ± 0.1 | 0.189 |

to be tested directly by NK3R antagonist injections into the ARC in follicular phase ewes. By contrast to the reduction in NK3R-IR cells that we observed in the ARC, no changes were seen in the POA or in any other hypothalamic nuclei analysed in the present study. We

were particularly surprised by the absence of any changes in the POA and RCh because senktide microinjections into either region are able to elicit a surge-like pattern of LH release (20) similar to that seen after i.c.v. injections of this agonist (6). Microimplants containing NKB antagonist (SB222200) into the RCh but not the POA reduce the amplitude of the LH surge by 40%, suggesting that NKB release in the RCh during the follicular phase is physiologically important to the generation of the LH surge (20). Because i.c.v. injections of kisspeptin antagonists in follicular phase ewes only reduce surge amplitude by 50% (22), it is tempting to speculate that NKB and kisspeptin act synergistically to elevate LH release during the surge. Interestingly, tract tracing data demonstrate that KNDy neurones receive direct input from neurones in the RCh (50) and administration of senktide into the RCh induces c-Fos expression in the ARC population (20). Taken together, these findings suggest that NKB signalling in the RCh plays a role in the preovulatory LH surge, and that the effect of NKB in the RCh is likely mediated, at least in part, by projections to ARC KNDy neurones. We are currently investigating the existence of reciprocal connection from the ARC to the RCh, constituting a potential pathway via which NKB/NK3R and kisspeptin/Kiss1r signalling are involved in the GnRH surge mechanism. If NKB/NK3R in KNDy cells contribute to this mechanism, then

administration of the NKB agonist, senktide, should, at least partially reverse the defects in GnRH surge amplitude seen in prenatal testosterone-treated ewes.

In the present study, as a marker for KNDy cells, we used kisspeptin immunoreactivity. Although the presence of KNDy cells has been confirmed in various species (9,14), it must be noted that single-labelled populations of NKB and/or kisspeptin cells have been documented in male humans (15) and mice (12,51). Therefore, we cannot exclude the possibility that we are overseeing potential changes in the co-localisation of NK3R and NKB-only cells. However, because previous work has shown that prenatal testosterone decreases NKB and dynorphin but not kisspeptin expression (33), we chose kisspeptin as a marker for KNDy cells because this peptide would not be affected by the treatment itself.

Although the above evidence supports a central role for NKB in regulating GnRH secretion, this influence in the ewe was assumed to be largely indirect, based on the complete absence of NK3R-IR co-localisation in ovine GnRH cells (11). Instead, the stimulatory influence of NKB in KNDy cells on GnRH secretion is considered to be conveyed by kisspeptin as an output signal, acting upon either GnRH cell bodies or terminals (13). Evidence for this upstream site of action originates from studies in which kisspeptin antagonists have been shown to block the stimulatory effects of NKB or senktide (52), as well as studies in which desensitisation of the kisspeptin receptor blocks the stimulatory effect of senktide in monkeys (53), and the absence of the stimulatory effect of senktide in Kiss1r KO mice (54). The current working hypothesis of the mechanisms by which NKB acts as a stimulatory 'start' signal in the generation of GnRH pulses in the ewe posits this action occurring via reciprocal KNDy-KNDy inputs at the level of KNDy cell bodies. Our observation in the present study of NK3R-IR localisation in terminals that are presynaptic to GnRH cell bodies suggests another possibility: NKB release by KNDy terminals acts in an autoregulatory manner upon the same terminals contributing to the enhanced release of kisspeptin. However, because we did not co-localise NK3R with KNDy peptides in inputs contacting GnRH neurones, we cannot conclude that the presynaptic NK3R inputs that we observed arose from KNDy cells and, indeed, they may have originated from any of the number of other NK3R-IR cell populations. For example, the effects of senktide injections in the POA on LH secretion (20) may be mediated either by actions on NK3R-containing cell bodies in that region, or by presynaptic NK3R in contact with POA GnRH neurones. Because we found very little co-localisation (approximately 5%) of NK3R within kisspeptin cells of the POA, it is possible that senktide effects on LH secretion from injections into this area are independent of kisspeptin, and are mediated instead by other transmitters/peptides. It is noteworthy that the effects of senktide on GnRH release in tissue slices of the mouse median eminence are also independent of kisspeptin (55) and because GnRH neurones in the mouse (12), similar to the sheep (11), lack NK3R, it is possible that the effect of senktide in the median eminence is also mediated by presynaptic actions of NKB, in this case via axo-axonic contacts. The possibility of presynaptic actions of NKB is supported by evidence in other systems and species; for example, in the rat striatum, in which tachykinins

presynaptically stimulate the release of dopamine (56). Finally, we would note that the observations reported in the present study are based on control animals; the possibility that changes in presynaptic localisation of NK3R are present in prenatal testosterone female sheep and contribute to reproductive neuroendocrine defects remains to be examined.

In summary, the decreases in NK3R that we observed in the ARC of prenatal testosterone-treated ewes complement previous observations of decreases in NKB and dynorphin peptides in KNDy cells (33) and suggest that the combined reduction in ligand and receptor components of NKB/NK3R signalling may contribute to alterations in the control of pulsatile or surge modes of GnRH/LH secretion. The constellation of adult reproductive dysfunction, as well as metabolic defects, in prenatal testosterone-treated ewes is very similar to that observed in women with polycystic ovarian syndrome (PCOS) (29) suggesting that the prenatal testosterone ewe may serve as a model for this disease (28). KNDy cells are present in the human female infundibular nucleus (equivalent to the ARC in ewes) and show morphological changes with loss of steroid feedback regulation of GnRH/LH (57,58). For example, in the infundibular nucleus of postmenopausal women, NKB gene expression is elevated as a result of reduced oestrogen negative-feedback (59). Thus, we would speculate that alterations in NKB/NK3R signalling may be, at least in part, responsible for the ovulatory defects observed in patients with PCOS. The prenatal testosterone-treated ewe could serve as an important translational model to test this hypothesis, with regard to the feedback control of GnRH/LH pulses, as well as the generation of the preovulatory LH surge.

## Acknowledgements

## References

1 Mussap CJ, Geraghty DP, Burcher E. Tachykinin receptors: a radioligand binding perspective. *J Neurochem* 1993; **60**: 1987–2009.

2 Almeida TA, Rojo J, Nieto PM, Pinto FM, Hernandez M, Martin JD, Candenas ML. Tachykinins and tachykinin receptors: structure and activity relationships. *Curr Med Chem* 2004; **11**: 2045–2081.

3 Chawla MK, Gutierrez GM, Young WS III, McMullen NT, Rance NE. Localization of neurons expressing substance P and neurokinin B gene transcripts in the human hypothalamus and basal forebrain. *J Comp Neurol* 1997; **384**: 429–442.

4 Topaloglu AK, Reimann F, Guclu M, Yalin AS, Kotan LD, Porter KM, Serin A, Mungan NO, Cook JR, Ozbek MN, Imamoglu S, Akalin NS, Yuksel B, O'Rahilly S, Semple RK. TAC3 and TACR3 mutations in familial hypogonadotropic hypogonadism reveal a key role for neurokinin B in the central control of reproduction. *Nat Genet* 2009; **41**: 354–358.

5 Young J, Bouligand J, Francou B, Raffin-Sanson ML, Gaillez S, Jeanpierre M, Grynberg M, Kamenicky P, Chanson P, Brailly-Tabard S, Guiochon-

Mantel A. TAC3 and TACR3 defects cause hypothalamic congenital hypogonadotropic hypogonadism in humans. *J Clin Endocrinol Metab* 2010; **95**: 2287–2295.

6  Billings HJ, Connors JM, Altman SN, Hileman SM, Holaskova I, Lehman MN, McManus CJ, Nestor CC, Jacobs BH, Goodman RL. Neurokinin B acts via the neurokinin-3 receptor in the retrochiasmatic area to stimulate luteinizing hormone secretion in sheep. *Endocrinology* 2010; **151**: 3836–3846.

7  Wakabayashi Y, Nakada T, Murata K, Ohkura S, Mogi K, Navarro VM, Clifton DK, Mori Y, Tsukamura H, Maeda K, Steiner RA, Okamura H. Neurokinin B and dynorphin A in kisspeptin neurons of the arcuate nucleus participate in generation of periodic oscillation of neural activity driving pulsatile gonadotropin-releasing hormone secretion in the goat. *J Neurosci* 2011; **30**: 3124–3132.

8  Ramaswamy S, Seminara SB, Ali B, Ciofi P, Amin NA, Plant TM. Neurokinin B stimulates GnRH release in the male monkey (*Macaca mulatta*) and is colocalized with kisspeptin in the arcuate nucleus. *Endocrinology* 2010; **151**: 4494–4503.

9  Navarro VM, Castellano JM, McConkey SM, Pineda R, Ruiz-Pino F, Pinilla L, Clifton DK, Tena-Sempere M, Steiner RA. Interactions between kisspeptin and neurokinin B in the control of GnRH secretion in the female rat. *Am J Physiol Endocrinol Metab* 2011; **300**: E202–E210.

10  Krajewski SJ, Anderson MJ, Iles-Shih L, Chen KJ, Urbanski HF, Rance NE. Morphologic evidence that neurokinin B modulates gonadotropin-releasing hormone secretion via neurokinin 3 receptors in the rat median eminence. *J Comp Neurol* 2005; **489**: 372–386.

11  Amstalden M, Coolen LM, Hemmerle AM, Billings HJ, Connors JM, Goodman RL, Lehman MN. Neurokinin 3 receptor immunoreactivity in the septal region, preoptic area and hypothalamus of the female sheep: co-localisation in neurokinin B cells of the arcuate nucleus but not in gonadotrophin-releasing hormone neurones. *J Neuroendocrinol* 2009; **22**: 1–12.

12  Navarro VM, Gottsch ML, Wu M, Garcia-Galiano D, Hobbs SJ, Bosch MA, Pinilla L, Clifton DK, Dearth A, Ronnekleiv OK, Braun RE, Palmiter RD, Tena-Sempere M, Alreja M, Steiner RA. Regulation of NKB pathways and their roles in the control of Kiss1 neurons in the arcuate nucleus of the male mouse. *Endocrinology* 2011; **152**: 4265–4275.

13  Lehman MN, Coolen LM, Goodman RL. Minireview: kisspeptin/neurokinin B/dynorphin (KNDy) cells of the arcuate nucleus: a central node in the control of gonadotropin-releasing hormone secretion. *Endocrinology* 2010; **151**: 3479–3489.

14  Goodman RL, Lehman MN. Kisspeptin neurons from mice to men: similarities and differences. *Endocrinology* 2012; **153**: 5105–5118.

15  Hrabovszky E, Sipos MT, Molnar CS, Ciofi P, Borsay BA, Gergely P, Herczeg L, Bloom SR, Ghatei MA, Dhillo WS, Liposits Z. Low degree of overlap between kisspeptin, neurokinin B, and dynorphin immunoreactivities in the infundibular nucleus of young male human subjects challenges the KNDy neuron concept. *Endocrinology* 2012; **153**: 4978–4989.

16  Goodman RL, Hileman SM, Nestor CC, Porter KL, Connors JM, Hardy SL, Millar RP, Cernea M, Coolen LM, Lehman MN. Kisspeptin, neurokinin B, and dynorphin act in the arcuate nucleus to control activity of the GnRH pulse generator in ewes. *Endocrinology* 2013; **154**: 4259–4269.

17  Caraty A, Fabre-Nys C, Delaleu B, Locatelli A, Bruneau G, Karsch FJ, Herbison A. Evidence that the mediobasal hypothalamus is the primary site of action of estradiol in inducing the preovulatory gonadotropin releasing hormone surge in the ewe. *Endocrinology* 1998; **139**: 1752–1760.

18  Merkley CM, Porter KL, Coolen LM, Hileman SM, Billings HJ, Drews S, Goodman RL, Lehman MN. KNDy (kisspeptin/neurokinin B/dynorphin) neurons are activated during both pulsatile and surge secretion of LH in the ewe. *Endocrinology* 2012; **153**: 5406–5414.

19  Fergani C, Routly JE, Jones DN, Pickavance LC, Smith RF, Dobson H. Kisspeptin, c-Fos and CRFR type 2 expression in the preoptic area and mediobasal hypothalamus during the follicular phase of intact ewes, and alteration after LPS. *Physiol Behav* 2013; **110–111**: 158–168.

20  Porter KL, Hileman SM, Hardy SL, Goodman RL. *Neurokinin B Signaling in the Retrochiasmatic Area is Essential for the Full Preovulatory LH Surge in Ewes*. San Diego, CA: Society for Neuroscience, 2013.

21  Estrada KM, Clay CM, Pompolo S, Smith JT, Clarke IJ. Elevated KiSS-1 expression in the arcuate nucleus prior to the cyclic preovulatory gonadotrophin-releasing hormone/lutenising hormone surge in the ewe suggests a stimulatory role for kisspeptin in oestrogen-positive feedback. *J Neuroendocrinol* 2006; **18**: 806–809.

22  Smith JT, Li Q, Pereira A, Clarke IJ. Kisspeptin neurons in the ovine arcuate nucleus and preoptic area are involved in the preovulatory luteinizing hormone surge. *Endocrinology* 2009; **150**: 5530–5538.

23  Pineda R, Garcia-Galiano D, Roseweir A, Romero M, Sanchez-Garrido MA, Ruiz-Pino F, Morgan K, Pinilla L, Millar RP, Tena-Sempere M. Critical roles of kisspeptins in female puberty and preovulatory gonadotropin surges as revealed by a novel antagonist. *Endocrinology* 2010; **151**: 722–730.

24  Smith JT, Li Q, Sing Yap K, Shahab M, Roseweir AK, Millar RP, Clarke IJ. Kisspeptin is essential for the full preovulatory LH surge and stimulates GnRH release from the isolated ovine median eminence. *Endocrinology* 2011; **152**: 1001–1012.

25  Oakley AE, Clifton DK, Steiner RA. Kisspeptin signaling in the brain. *Endocr Rev* 2009; **30**: 713–743.

26  Gorski RA. Sexual dimorphisms of the brain. *J Anim Sci* 1985; **61**(Suppl. 3): 38–61.

27  Simerly RB. Wired for reproduction: organization and development of sexually dimorphic circuits in the mammalian forebrain. *Annu Rev Neurosci* 2002; **25**: 507–536.

28  Padmanabhan V, Veiga-Lopez A. Sheep models of polycystic ovary syndrome phenotype. *Mol Cell Endocrinol* 2013; **373**: 8–20.

29  Padmanabhan V, Sarma HN, Savabieasfahani M, Steckler TL, Veiga-Lopez A. Developmental reprogramming of reproductive and metabolic dysfunction in sheep: native steroids vs. environmental steroid receptor modulators. *Int J Androl* 2010; **33**: 394–404.

30  Sharma TP, Herkimer C, West C, Ye W, Birch R, Robinson JE, Foster DL, Padmanabhan V. Fetal programming: prenatal androgen disrupts positive feedback actions of estradiol but does not affect timing of puberty in female sheep. *Biol Reprod* 2002; **66**: 924–933.

31  Veiga-Lopez A, Astapova OI, Aizenberg EF, Lee JS, Padmanabhan V. Developmental programming: contribution of prenatal androgen and estrogen to estradiol feedback systems and periovulatory hormonal dynamics in sheep. *Biol Reprod* 2009; **80**: 718–725.

32  Herbosa CG, Dahl GE, Evans NP, Pelt J, Wood RI, Foster DL. Sexual differentiation of the surge mode of gonadotropin secretion: prenatal androgens abolish the gonadotropin-releasing hormone surge in the sheep. *J Neuroendocrinol* 1996; **8**: 627–633.

33  Cheng G, Coolen LM, Padmanabhan V, Goodman RL, Lehman MN. The kisspeptin/neurokinin B/dynorphin (KNDy) cell population of the arcuate nucleus: sex differences and effects of prenatal testosterone in sheep. *Endocrinology* 2010; **151**: 301–311.

34  Jackson LM, Timmer KM, Foster DL. Organizational actions of postnatal estradiol in female sheep treated prenatally with testosterone: programming of prepubertal neuroendocrine function and the onset of puberty. *Endocrinology* 2009; **150**: 2317–2324.

35  Jackson LM, Timmer KM, Foster DL. Sexual differentiation of the external genitalia and the timing of puberty in the presence of an antiandrogen in sheep. *Endocrinology* 2008; **149**: 4200–4208.

36  Veiga-Lopez A, Steckler TL, Abbott DH, Welch KB, MohanKumar PS, Phillips DJ, Refsal K, Padmanabhan V. Developmental programming: impact of excess prenatal testosterone on intrauterine fetal endocrine milieu and growth in sheep. *Biol Reprod* 2011; **84**: 87–96.

37 Veiga-Lopez A, Wurst AK, Steckler TL, Ye W, Padmanabhan V. Developmental programming: postnatal estradiol amplifies ovarian follicular defects induced by fetal exposure to excess testosterone and dihydrotestosterone in sheep. *Reprod Sci* 2014; **21**: 444–455.

38 Jackson LM, Mytinger A, Roberts EK, Lee TM, Foster DL, Padmanabhan V, Jansen HT. Developmental programming: postnatal steroids complete prenatal steroid actions to differentially organize the GnRH surge mechanism and reproductive behavior in female sheep. *Endocrinology* 2013; **154**: 1612–1623.

39 Veiga-Lopez A, Ye W, Phillips DJ, Herkimer C, Knight PG, Padmanabhan V. Developmental programming: deficits in reproductive hormone dynamics and ovulatory outcomes in prenatal, testosterone-treated sheep. *Biol Reprod* 2008; **78**: 636–647.

40 Robinson JE, Birch RA, Foster DL, Padmanabhan V. Prenatal exposure of the ovine fetus to androgens sexually differentiates the steroid feedback mechanisms that control gonadotropin releasing hormone secretion and disrupts ovarian cycles. *Arch Sex Behav* 2002; **31**: 35–41.

41 Karsch FJ, Foster DL, Legan SJ, Ryan KD, Peter GK. Control of the preovulatory endocrine events in the ewe: interrelationship of estradiol, progesterone, and luteinizing hormone. *Endocrinology* 1979; **105**: 421–426.

42 Watson RE Jr, Wiegand SJ, Clough RW, Hoffman GE. Use of cryoprotectant to maintain long-term peptide immunoreactivity and tissue morphology. *Peptides* 1986; **7**: 155–159.

43 Hunyady B, Krempels K, Harta G, Mezey E. Immunohistochemical signal amplification by catalyzed reporter deposition and its application in double immunostaining. *J Histochem Cytochem* 1996; **44**: 1353–1362.

44 Franceschini I, Lomet D, Cateau M, Delsol G, Tillet Y, Caraty A. Kisspeptin immunoreactive cells of the ovine preoptic area and arcuate nucleus co-express estrogen receptor alpha. *Neurosci Lett* 2006; **401**: 225–230.

45 Cheng G, Coolen LM, Padmanabhan V, Goodman RL, Lehman MN. The kisspeptin/neurokinin B/dynorphin (KNDy) cell population of the arcuate nucleus: sex differences and effects of prenatal testosterone in sheep. *Endocrinology* 2009; **151**: 301–311.

46 Goubillon ML, Forsdike RA, Robinson JE, Ciofi P, Caraty A, Herbison AE. Identification of neurokinin B-expressing neurons as an highly estrogen-receptive, sexually dimorphic cell group in the ovine arcuate nucleus. *Endocrinology* 2000; **141**: 4218–4225.

47 Goodman RL, Coolen LM, Lehman MN. A role for neurokinin B in pulsatile GnRH secretion in the ewe. *Neuroendocrinology* 2014; **99**: 18–32.

48 Mittelman-Smith MA, Williams H, Krajewski-Hall SJ, Lai J, Ciofi P, McMullen NT, Rance NE. Arcuate kisspeptin/neurokinin B/dynorphin (KNDy) neurons mediate the estrogen suppression of gonadotropin secretion and body weight. *Endocrinology* 2012; **153**: 2800–2812.

49 Yan X, Yuan C, Zhao N, Cui Y, Liu J. Prenatal androgen excess enhances stimulation of the GNRH pulse in pubertal female rats. *J Endocrinol* 2014; **222**: 73–85.

50 Coolen LM, Smith TG, Lehman MN, Hileman SM, Connors JM, Goodman RL. *Arcuate KNDy Neurons Receive Afferent Projections from the Retrochiasmatic Area in the Ewe*. San Diego, CA: Society for Neuroscience, 2013.

51 Navarro VM, Gottsch ML, Chavkin C, Okamura H, Clifton DK, Steiner RA. Regulation of gonadotropin-releasing hormone secretion by kisspeptin/dynorphin/neurokinin B neurons in the arcuate nucleus of the mouse. *J Neurosci* 2009; **29**: 11859–11866.

52 Grachev P, Li XF, Lin YS, Hu MH, Elsamani L, Paterson SJ, Millar RP, Lightman SL, O'Byrne KT. GPR54-dependent stimulation of luteinizing hormone secretion by neurokinin B in prepubertal rats. *PLoS ONE* 2012; **7**: e44344.

53 Ramaswamy S, Seminara SB, Plant TM. Evidence from the agonadal juvenile male rhesus monkey (*Macaca mulatta*) for the view that the action of neurokinin B to trigger gonadotropin-releasing hormone release is upstream from the kisspeptin receptor. *Neuroendocrinology* 2011; **94**: 237–245.

54 Garcia-Galiano D, van Ingen Schenau D, Leon S, Krajnc-Franken MA, Manfredi-Lozano M, Romero-Ruiz A, Navarro VM, Gaytan F, van Noort Pl, Pinilla L, Blomenrohr M, Tena-Sempere M. Kisspeptin signaling is indispensable for neurokinin B, but not glutamate, stimulation of gonadotropin secretion in mice. *Endocrinology* 2012; **153**: 316–328.

55 Gaskins GT, Glanowska KM, Moenter SM. Activation of neurokinin 3 receptors stimulates GnRH release in a location-dependent but kisspeptin-independent manner in adult mice. *Endocrinology* 2013; **154**: 3984–3989.

56 Glowinski J, Kemel ML, Desban M, Gauchy C, Lavielle S, Chassaing G, Beaujouan JC, Tremblay L. Distinct presynaptic control of dopamine release in striosomal- and matrix-enriched areas of the rat striatum by selective agonists of NK1, NK2 and NK3 tachykinin receptors. *Regul Pept* 1993; **46**: 124–128.

57 Rance NE. Menopause and the human hypothalamus: evidence for the role of kisspeptin/neurokinin B neurons in the regulation of estrogen negative feedback. *Peptides* 2009; **30**: 111–122.

58 Rance NE, Young WS III. Hypertrophy and increased gene expression of neurons containing neurokinin-B and substance-P messenger ribonucleic acids in the hypothalami of postmenopausal women. *Endocrinology* 1991; **128**: 2239–2247.

59 Rance NE, Krajewski SJ, Smith MA, Cholanian M, Dacks PA. Neurokinin B and the hypothalamic regulation of reproduction. *Brain Res* 2010; **1364**: 116–128.