1 **Prenatal Testosterone Excess Decreases Neurokinin 3 Receptor**

2 **Immunoreactivity within the Arcuate Nucleus KNDy cell population.**

3

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14

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Abstract

Prenatal exposure of the female ovine fetus to excess testosterone (T) leads to neuroendocrine disruptions in adulthood, evidenced by defects in responsiveness to the ability of gonadal steroids to regulate 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 T-treatment. KNDy cells also colocalise 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 signaling may be involved in the positive feedback actions of oestradiol leading to the GnRH/LH surge in the ewe. Thus, we hypothesise that decreased expression of NK3R in KNDy cells may be present in the brains of prenatal T-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 T-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 colocalised 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 colocalization in GnRH neurones in either the POA or MBH, >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, decreased NK3R within KNDy cells in prenatal T-treated sheep complement 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.

Introduction

A candidate afferent signaling system that has received much recent attention in the central control of GnRH secretion is that comprising the tachykinin neurokinin B (NKB) and its high affinity receptor neurokinin-3 (NK3R) (4, 5). Although NKB/NK3R signaling is implicated in diverse physiological functions (6), 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, (encoded by *TAC3R*), displayed hypogonadotrophic hypogonadism and infertility (7, 8). Since then, a growing number of animal studies have established a close association between NKB/NK3R signaling and GnRH/LH secretion in species including the sheep (9), goat (10), primate (11) and rat (12). However, the precise neuronal pathway(s), via which NKB stimulates GnRH secretion, are not yet fully determined. While a subset of GnRH neurones in the rat have been shown to colocalise NK3R (13), similar studies in sheep (14) and mice (15) have failed to reveal NK3R in GnRH neurones, suggesting that NKB action upon GnRH secretion is likely exerted via inputs from other neurones, either directly or indirectly.

The neuroanatomical location of NK3R has been previously described 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) (14). In the ARC, NKB is colocalised with two other neuropeptides, kisspeptin and dynorphin in a population that are termed KNDy (Kisspeptin, Neurokinin B and Dynorphin) cells (16). KNDy cells are present in the ARC of all species studied to date (17), and are thought to play a key role in the negative feedback effects of oestradiol and progesterone upon GnRH (17). In addition, KNDy cells are thought to comprise a critical component of the circuitry responsible for the generation of GnRH/LH pulses (10, 12, 19). Accumulating evidence suggests that NKB acts as an auto-regulatory signal within the network of reciprocally interconnected KNDY cells, a signal that is responsible for the initiation of each GnRH pulse (19).

In addition to its role in negative feedback, in the sheep, NKB/NK3R signaling may also be important in the generation of the preovulatory GnRH/LH surge (16, 20-22). Intracerebroventricular (icv) microinjections of senktide, a NK3R specific

100   agonist (5), results in a surge-like elevation of LH during the follicular but not the
101   luteal phase of the ovine oestrous cycle (9). Bilateral senktide microinjections into
102   the RCh (9) and POA (23) are each able to produce a similar surge-like elevation
103   of LH suggesting that these two areas, each of which contain NK3R-positive cells
104   may participate in the control of the LH surge. In addition, there are several lines
105   of evidence suggesting that KNDy cells may participate in the GnRH/LH surge in
106   the ewe (24-29). For example, in the sheep (20), unlike rodents (30), oestradiol
107   implants in the mediobasal hypothalamus, close to the vicinity of KNDy cells, are
108   sufficient to induce a GnRH/LH surge Thus NKB/NK3R signaling could potentially
109   play a role in both negative and positive feedback effects of gonadal steroids in the
110   sheep, acting at potential target sites that include the POA, RCh, and KNDy cells
111   of the ARC.

112   Responsiveness of the adult GnRH system to hormonal feedback controls is
113   programmed during development by events that include fetal exposure to
114   androgens (31, 32). While normal sexual differentiation depends on appropriate
115   timing of exposure of fetuses to androgens, exposure to excess androgens in
116   animal models can result in long-term deficits in reproductive functions at multiple
117   levels including the GnRH system (33). For example, exposure of female ovine
118   fetuses to excess testosterone (T) during days 30-90 of the 147 day gestation,
119   leads to neuroendocrine defects in the responsiveness of the GnRH system to both
120   negative and positive steroid feedback (34-37). KNDy neurones have been
121   implicated as critical mediators of the detrimental effects of prenatal T (38), and
122   prenatal T treatment results in dramatic alterations in KNDy peptides in the adult
123   ARC, with NKB and dynorphin being markedly reduced but kisspeptin remaining
124   unaltered. This peptide imbalance within a single neuronal population has been
125   hypothesised to underlie some of the defects in responsiveness of the GnRH system
126   to oestradiol and progesterone seen in adult female sheep exposed prenatally to
127   excess T (38).

128   Whether postsynaptic receptors for any of the KNDy peptides are similarly altered
129   in prenatal T-treated animals has not yet been examined, and given the evidence
130   for participation of NKB/NK3R signaling in both pulsatile and surge modes of
131   GnRH/LH secretion, we hypothesised that changes in NK3R expression in either
132   the ARC or in other regions where it has been shown to alter LH secretion (e.g.,
133   RCh, POA), may be present in the brains of prenatal T-treated female sheep. To

134   test this hypothesis, we first compared the overall number of NK3R-IR cells in the
135   ARC, RCh, POA and other hypothalamic nuclei between prenatal T-treated and
136   control animals. Second, we used dual-label immunofluorescence and confocal
137   microscopy to determine whether NK3R might be specifically altered within the
138   KNDy cell subpopulation of the ARC. Finally, since NK3R-IR is seen in fiber and
139   terminals as well as cell bodies, we explored the possibility that NKB might act
140   presynaptically to influence GnRH secretion by determining whether NK3R-IR
141   terminals in the preoptic area (POA) and medial basal hypothalamus (MBH) are in
142   direct synaptic contact with GnRH cell bodies in those regions. To control for the
143   possible influence of differences in circulating steroids between the experimental
144   and control groups, animals were ovariectomised prior to sacrifice and implanted
145   with hormonal regimens designed to produce late follicular phase concentrations
146   of oestradiol.

147

148 **Materials and Methods:**

149 **Animal Care and Treatment**
150 All procedures involving animals were approved by the University of Michigan Animal
151 Care and Use Committee and are consistent with National Research Council's Guide
152 for the Care and Use of Laboratory Animals. Experiments were conducted in 2-year
153 old control and prenatal T-treated Suffolk ewes during the breeding season. Housing,
154 breeding, lambing and maintenance took place at the Sheep Research Facility at the
155 University of Michigan (Ann Arbor, MI, 42°18′ north latitude) as has been previously
156 described (39, 40).

157 Pregnant ewes were administered intramuscular (im) injections of T propionate
158 (100mg/injection catalog item T1875; Sigma-Aldrich, St. Louis, MO; n=8) twice
159 weekly, suspended in cottonseed oil (catalog item C7767; Sigma-Aldrich, St.Louis,
160 MO, USA) in the hind leg from days 30–90 of pregnancy (term=147 days). The dose
161 of T propionate administered results in levels of T in the female fetus comparable to
162 those in fetal males (41). Control ewes received an equal volume of vehicle (2 ml
163 cottonseed oil; n=9) in the same regimen as T. Lambs were born in March/April. After
164 weaning, they were maintained outdoors under natural photoperiods with a daily
165 maintenance feeding and free access to water. During the first breeding season,
166 progesterone profiles and ultrasonographic assessment of ovarian status was carried

167 out (42). In addition, at approximately 19 months of age, all ewes were ovariectomised
168 (OVX) and evaluated for LH surge and sexual behavior profiles (43).For the present
169 study, and in order to normalise the hormonal milieu between all animals, a 1-cm-long
170 SILASTIC capsule (inner diameter, 3.35 mm and outer diameter, 4.65 mm; Dow
171 Corning Corp., Midland, MI) filled with 17β oestradiol (oestradiol; Sigma-Aldrich, St.
172 Louis, MO, USA) in addition to two controlled internal drug release (CIDR)
173 progesterone implants (InterAG, Hamilton, Waikato, New Zealand) were inserted
174 subcutaneously in to each animal. CIDRS were removed 14 days later and sequentially
175 all animals received additional four 3-cm-long oestradiol implants (see before) to
176 simulate ovarian steroid levels equivalent to a normal follicular phase as well as
177 generate a GnRH/LH surge (47). Animals were euthanised ~20 hours after insertion
178 of the oestradiol implants and specifically during the late follicular phase (i.e., prior to
179 the LH surge in both control or T-treated animals) (43).The oestradiol implant insertion
180 and euthanasia where staggered at hourly intervals to allow for the time needed to
181 perfuse and extract the brain.

182 **Tissue collection and preparation**
183 Ewes were injected intravenously (iv) twice at 10-minute intervals with 25,000 U of
184 heparin (catalog item 402588B; Abraxiz pharmaceutical Products, Schaumburg IL,
185 USA) and then deeply anaesthetised with iv sodium pentobarbital (2-3g; catalog item
186 P3761; Sigma-Aldrich, St. Louis, MO, USA). Animals were rapidly decapitated, and the
187 heads perfused via both internal carotids with 6 litres of 4% paraformaldehyde in 0.1
188 M phosphate buffer (PB; pH 7.3) mixed with 0.1 % sodium nitrite and administered
189 with 10 U/ml heparin. After perfusion, the brain was removed and a tissue block
190 containing the septal region, POA, and hypothalamus dissected out. Blocks were
191 incubated in 4% paraformaldehyde at 4 °C overnight for post-fixation and then
192 transferred into 30% sucrose in 0.1 M PB for cryoprotection until infiltration took place.
193 A sliding freezing microtome (Leica Biosystems, SM 200R, Walldorf, Germany) was
194 used to section frozen blocks of tissue containing POA and hypothalamus into 6 series
195 of coronal 45 μm slices. Free-floating sections were stored in cryoprotectant solution
196 (30% ethylene glycol, 1% polyvinylpyrrolidone, 30% sucrose in sodium phosphate
197 buffer; (48) at -20°C until processed for immunohistochemistry. Within each
198 experiment, tissue sections from all experimental groups were processed
199 simultaneously as described below. All immunohistochemical procedures were carried
200 out at room temperature under gentle agitation. Unless otherwise stated, tissue
201 sections were washed with 0.1 M phosphate buffer saline (PBS; pH 7.2) between steps.

202 Antibodies were diluted with blocking solution, comprised of 0.4% Triton X-100
203 (catalog item BP151-500, Sigma-Aldrich, St.Louis, MO, USA) and 4% normal goat
204 serum (NGS; catalog item H005-000-121, Jackson ImmunoResearch Laboratories,
205 West Grove, PA, USA) in 0.1M PBS.
206
207 **Experiment 1: Effects of prenatal T-treatment on NK3R-IR in the POA and**
208 **hypothalamus.**
209
210 **Single-label immunohistochemistry for NK3R**
211 The distribution and quantification of NK3R-IR cells was determined in a series of every
212 6th section (270 µm apart). Free-floating sections were washed thoroughly in 0.1 M
213 PBS for several hours to remove excess cryoprotectant followed by a 10 minute
214 incubation with PBS containing 1% hydrogen peroxide (H_2O_2; catalog item H325,
215 Fishers Scientific, Pittsburgh, PA, USA) to eliminate endogenous peroxidase activity.
216 Next, sections were incubated in blocking solution for 1 hour followed by an overnight
217 (17 hour) incubation with polyclonal rabbit anti-NK3R (1:10,000; catalog item NB300-
218 102, Novus Biological, Littleton, CO, USA). After incubation with the primary
219 antiserum, sections were incubated with biotinylated goat anti-rabbit IgG (1:500;
220 catalog item BA-1000; Vector laboratories, Burlingame, CA, USA) for 1 hour followed
221 by incubation with ABC reagent (1:500 diluted in 0.1 M PBS; avidin and biotinylated
222 horseradish peroxidase macromolecular complex, catalog item PK-6100; Vector
223 Laboratories) for 1 hour. NK3R labelling was visualised using 3,3'-diaminobenzidine
224 tetrahydrochloride (0.2 mg/ml) (DAB; Catalogue # D5905, Sigma-Aldrich, St.Louis,
225 MO, USA) with 0.00004% hydrogen peroxide in PB as substrate. Finally, they were
226 mounted onto Superfrost/Plus Microscope Slides (Fisher), air dried, and coverslipped
227 with DPX Mountant. Omission of NK3R antibody from the immunohistochemical
228 protocol resulted in complete absence of staining. Furthermore, preabsorption controls
229 with purified antigen have been previously performed and published (see below) (14).
230
231 **Experiment 2: Effects of prenatal T-treatment on NK3R-IR within the POA**
232 **kisspeptin and ARC KNDy cell population.**
233
234 **Dual-label immunofluorescent detection of NK3R and kisspeptin**
235 In order to determine if changes in NK3R-IR occurred specifically within the POA
236 kisspeptin or ARC KNDy cell population an alternate series of every 6th section (270

237 µm apart) containing the POA or ARC was processed for dual-label
238 immunofluorescence and confocal microscopic analysis. A modification of the protocol
239 used by Hunyady et al (1996) was carried out to eliminate possible cross-linking
240 between kisspeptin and NK3R antibodies (both raised in rabbits) and false
241 colocalization between antigens (49). Initially, free-floating tissue sections were
242 washed several hours in PBS for cryoprotectant removal. Thereafter, they were
243 incubated in PBS containing 1% H_2O_2 for 10 min followed by a 1 hour incubation in
244 blocking solution (with 20% NGS). Next, rabbit polyclonal anti-NK3R (1:10,000, for
245 17 hours) was applied. Sections were then incubated sequentially in biotinylated goat
246 anti-rabbit (1:500 for 1 hour) and ABC-elite solution (1:500 diluted in 0.1 M PBS, for
247 1 hour). Following amplification with TSA^{TM} Biotin system Biotinyl Tyramide agent
248 (1:250 diluted in 0.1 M PBS with 3% H_2O_2; catalogue item NEL700A001KT,
249 PerkinElmer Life Sciences, Waltham, MA, USA), NK3R was visualised with Alexa 488
250 conjugated streptavidin (1:100 diluted in 0.1 M PBS, for 30 min; catalogue item S-
251 32354 Invitrogen/Molecular Probe, Eugene, OR). Sections were then processed for
252 detection of kisspeptin. First, they were incubated for 17 hours with primary antibody
253 rabbit anti-Kisspeptin (gift from A. Caraty, Universite Tours, Nouzilly, France, lot
254 number 564) at a dilution of 1:2,000 (for POA sections) or 1:10,000 (for ARC sections)
255 and visualised with goat anti-rabbit Alexa 555 (1:100 in 0.1 M PBS, for 30 min;
256 catalogue item A-21428, Invitrogen/Molecular Probe, Eugene, OR). Finally, sections
257 were mounted on glass slides, dried and coverslipped with mount medium gelvatol.
258 Control sections for the dual immunofluorescent procedure included omission of each
259 of the primary antibodies from the immunostaining protocol, which resulted in a
260 complete absence of staining for the corresponding antigen. In addition, pre-
261 absorption controls have been performed for each of the antibodies in previous studies
262 (14) in each case pre-incubation of the diluted antiserum with nanomolar
263 concentrations of purified antigen was shown to be sufficient to eliminate all specific
264 staining in ewe hypothalamic sections. Finally, the kisspeptin antibody used has been
265 shown to be specific for kisspeptin cells of the ovine brain and not to cross-react with
266 other RFamide peptides (50).
267
268 **Experiment 3: Identification of pre-synaptic NK3R terminals onto GnRH**
269 **neurones in the POA and MBH**
270
271 **Triple-label immunofluorescent detection of GnRH, NK3R and Synaptophysin**

272   A series of every 12th section (540 μm apart) through the POA and MBH were used for
273   GnRH/NK3R/Synaptophysin triple labelling. Similar to the protocols described above,
274   free-floating sections were washed in 0.1 M PBS for several hours in order to remove
275   cryoprotectant. Next, they were incubated in 1% H_2O_2 diluted in PBS for 10 minutes,
276   followed by a 1 hour incubation in blocking solution (with 20% NGS). Thereafter,
277   sections were incubated sequentially in rabbit polyclonal anti-NK3R, biotinylated goat
278   anti-rabbit, ABC-elite solution and TSA^{TM} Biotin system Biotinyl Tyramide agent, as
279   described above. NK3R was visualised with Alexa 488 conjugated streptavidin (1:100
280   in 0.1 M PBS, for 30 min). The second primary antibody, rabbit anti-GnRH (1:1,000;
281   LR-5, gift from R. Benoit, Montréal General Hospital, Montréal, Canada), was visualised
282   using indirect detection with goat anti-rabbit Alexa 555 (1:100 in 0.1 M PBS; catalogue
283   item S-32354, Invitrogen/Molecular Probe, Eugene, OR). During the GnRH antibody
284   incubation period, mouse anti-synaptophysin (1:200; catalogue item S5768; Sigma-
285   Aldrich, St. Louis, MO, USA) was also co-incubated and visualised with Donkey anti-
286   Mouse Cy5 (1:100 in 0.1 M PBS, for 30 min; Catalogue item 715175151, Jackson
287   Immunoresearch West Grove, PA). Controls omitting one, two or all three primary
288   antisera from the protocol completely eliminated all specific staining for the
289   corresponding antigen(s).
290
291   **Data analysis**
292   For single-label NK3R, the distribution of IR cells and fibers was examined in sections
293   through the POA and hypothalamus of each ewe. Three representative sections of the
294   rostral, middle, and caudal divisions of the ARC, retrochiasmatic area (Rch), ventral
295   portion premammillary nucleus (PMv), preoptic area (POA), lateral hypothalamic area
296   (LHA), paraventricular nucleus (PVN) were quantitatively analyzed per animal in each
297   group. Each nucleus was determined by its cytoarchitectonic boundaries and all cells
298   within those boundaries were quantified. Areas chosen for analysis were based on the
299   regional distribution of NK3R-IR cells previously described in the ewe (14). The ARC,
300   which contains prominent NK3R-IR, was divided in to 3 rostral-caudal divisions for
301   more detailed analysis in this study as previously (14, 51). Our preliminary
302   observations revealed that the rostral ARC contained very few NK3R-IR cells and fibers
303   compared to the middle and the caudal ARC. Moreover, given that a large majority of
304   KNDy cells are found in the middle and caudal divisions (51, 52), we selected these
305   sub-regions for detailed comparison between control and prenatal T-treated animals.

306 For single-label analyses (Experiment 1), NK3R-IR cells were examined and quantified

307 with a Leica DMRD microscope (Leica Microsystems GmbH, Wetzlar, Germany) and

308 identified by the presence of dense reaction product that labelled their somas and

309 dendrites. Images were captured using a digital camera (Magnafire; Optronics, Goleta,

310 CA, USA) attached to the microscope and imported in to Adobe Photoshop 7.0 (Adobe

311 Systems, San Jose, CA, USA). Photomicrographs were not altered in any way except

312 for minor adjustments of brightness and contrast.

313 Sections processed for dual and triple immunofluorescence were analyzed using a

314 Zeiss LSM-510 laser-scanning confocal microscope system (Zeiss, Thronwood, NY).

315 Alexa 488 fluorescence was visualised and imaged with a 505-530 nm emission filter

316 and Argon laser whereas Alexa 555 and CY5 fluorescence with a 560-nm and 680-nm

317 emission filter and a HeNe laser. Confocal Z-stacks of optical sections (1 μm at 63X

318 magnification) were captured through NK3R, Kisspeptin and GnRH-IR neurones. Three

319 Z stacks from the middle and caudal ARC of each animal were used for analysis of

320 NK3R/Kisspeptin colocalization. A total of 700 kisspeptin-IR cells from the mARC and

321 692 cells from cARC (between 38-42 kisspeptin-IR cells per treatment group and ARC

322 subdivision) were analyzed. For examination of possible colocalization in the POA, a

323 total of 42 kisspeptin-IR cells from 3 control animals (between 12-16 kisspeptin-IR

324 cells per animal) were analyzed.

325 For analysis of GnRH/NK3R/Synaptophysin material, 6-10 Z-stacks were captured

326 from the POA and ARC to gather sufficient number of GnRH-IR neurones for analysis.

327 Putative contacts between NK3R/synaptophysin-positive terminals and GnRH-IR

328 somas were defined as a direct apposition without any intervening (black) pixels. A

329 total 49 POA GnRH neurones and 34 MBH GnRH neurones were analyzed from 5

330 random control animals (between 7-12 POA and 6-7 MBH GnRH neurones per animal).

331 First, the somal perimetre was calculated by tracing the neurone. Thereafter, in each

332 z-stack of 1 μm optical section, the number of NK3R-positive terminals in direct contact

333 with the GnRH neurone was determined. The percentage of GnRH neurones in the POA

334 and MBH having one or more NK3R-positive contacts was calculated, as was the mean

335 number of NK3R-positive contacts onto GnRH somas per animal, and the mean number

336 of contacts per 10 μm of GnRH somal perimeter.

337

338 **Statistical Analysis**

339   All data are expressed as the mean ± standard error of mean (SEM). Statistical

340   comparison between control and prenatal T-treated ewes (Experiments 1 and 2), and

341   between brain regions (Experiment 3) were assessed with a Student t-test. All

342   statistics were done using Sigma Stat for windows (SPSS Inc., Chicago, Illinois, USA)

343   and a *P* value of less than 0.05 was considered significant in all analyses.

344

345   **Results**

346   **Experiment 1: Effects of prenatal T-treatment on NK3R-IR cell number in the**

347   **POA and hypothalamus.**

348   NK3R-IR cells were present in a number of areas of the hypothalamus in addition to

349   the ARC, as depicted in Fig. 1. The most prominent and dense populations of NK3R-

350   IR neurones, other than the ARC, were observed in the following regions (in

351   descending order of overall cell number): the hypothalamic paraventricular nucleus

352   (PVN), lateral hypothalamic area (LHA), ventral premammillary nucleus (PMv), Rch,

353   and POA. In the ARC, where KNDY cells reside, we confirmed a large number of NK3R-

354   IR cells, specifically in the middle and caudal divisions of this nucleus (Fig. 1).

355   Quantitative cell counts revealed that the mean number of NK3R-IR cells observed in

356   the ARC of control ewes was significantly greater than that of prenatal T-treated

357   animals in both the middle (control: 53.8 ± 2.9 *vs.* prenatal T: 41.6 ± 2.8; P=0.009)

358   and caudal portions (control: 42.7 ± 4.0 *vs.* prenatal T: 30.0 ± 2.5; P= 0.019; Fig. 2)

359   of this nucleus. No significant differences in NK3R-IR cell number between control and

360   prenatal T-treated animals were observed in any of the other nuclei or areas analyzed

361   (Fig. 2).

362

363   **Experiment 2: Effect of prenatal T-treatment on NK3R-IR colocalization**

364   **within the ARC KNDy cell population.**

365   To determine whether changes in NK3R-IR cell number observed in the ARC, reflect a

366   change in NK3R specifically in the KNDy cell population, we analyzed sections

367   processed for dual-immunofluorescent localization of NK3R and kisspeptin (Kiss).

368   Prenatal T-treated animals showed a decrease in the number of dual-labelled

369   NK3R/Kiss cells (control: 19.4 ± 1.7 *vs.* prenatal T: 14.4 ± 1.2; P=0.049; Fig. 3G) as

370   well as the total number (single-labelled + dual-labelled) of ARC NK3R-IR cells

371   (control: 25.9 ± 2.1 *vs.* prenatal T: 20.7 ± 1.7; P=0.021 Fig. 3G). As in previous

372   studies (38), we saw no difference between control and prenatal T animals in the total

373 number of Kiss cells (Fig. 3G), and consistent with the decrease in number of dual
374 NK3R/Kiss cells, the number of single-labelled Kiss cells was significantly higher in
375 prenatal T ewes (control: 21.5 ± 1.6 *vs.* prenatal T: 26.7 ± 1.4; P=0.038; Fig. 3G).

376 We used the numbers of dual-labelled and total cells in individual animals to calculate
377 the percentage of ARC Kiss-IR cells co-localizing NK3R, and, conversely, the
378 percentage of NK3R-IR neurones co-localizing Kiss. The mean percentage of Kiss-IR
379 neurones co-localizing NK3R was significantly decreased in prenatal T animals
380 compared to controls (control: 47.1 ± 3.0% *vs.* prenatal T: 34.7 ± 2.4%; P=0.005;
381 Fig. 3H). By contrast, there was no significant difference between control and prenatal
382 T-treated animals in the percentage of NK3R-IR neurones co-localizing Kiss (Fig. 3H).

383 Since NK3R-IR cells are present in the POA (Figs. 1 and 2), we also examined
384 kisspeptin cells in the ovine POA for colocalization of NK3R. However, the
385 kisspeptin/NK3R colocalization in the POA was infrequent and variable (5.3 ± 5.3%,
386 mean ± S.E.M.) so that further comparison with prenatal T-treated animals was not
387 pursued.
388
389 **Experiment 3: Colocalization of NK3R-IR in presynaptic terminals contacting**
390 **GnRH neurones**
391 In addition to detecting NK3R-IR in cell bodies (Experiment 1), we also noted NK3R
392 localization in fibers and terminals throughout a number of hypothalamic regions
393 including the POA and mediobasal hypothalamus (MBH). Consequently we processed
394 sections for triple-label detection of NK3R, GnRH, and synaptophysin to determine
395 whether any of these NK3R-positive terminals were directly presynaptic to GnRH cell
396 bodies in either the POA or MBH. Examination of triple-labelled sections showed that
397 from a total of 83 GnRH cells analyzed (49 in the POA, and 34 in the MBH), none
398 contained NK3R, confirming our earlier results showing the lack of colocalization of
399 NK3R in ovine GnRH cells (14). However, NK3R-positive fibers were observed adjacent
400 to, and intermixed with, GnRH cells and dendrites in both the POA and MBH. We found
401 that >70 % of GnRH neurones examined were contacted by one or more NK3R-IR
402 presynaptic bouton (defined by the colocalization of synaptophysin; Fig. 4, Table 1).
403 Neither the percentage of GnRH cells receiving inputs, nor the mean number of inputs,
404 varied regionally between the POA and MBH (Table 1). Similarly, the mean number of
405 contacts per 10µm GnRH somal perimeter did not differ between the POA and MBH
406 (Table 1).

407

Discussion

409 Our results indicate that prenatal T-treated ewes show significantly diminished
410 numbers of NK3R-IR neurones in the ARC compared to control animals. Furthermore,
411 the decrease in NK3R was primarily due to changes within the KNDy cell population
412 and not in other ARC cells as the number of single-labelled NK3R-IR cells (i.e., NK3R
413 cells outside the KNDy cell population) in this region showed no difference between
414 control and prenatal T animals. The reduced number of NK3R-IR cells observed in
415 prenatal T female sheep parallels the decrease in numbers of NKB cells previously
416 observed in this animal model (38) and suggests that the combined decrease in both
417 ligand and receptor may contribute to defects in the control of GnRH/LH secretion.

418 Two possible functional consequences may be envisaged. One rests upon the proposed
419 role of NKB/NK3R signaling in the generation of GnRH pulses (16). In the current
420 model of GnRH pulse generation in ruminants (53), NKB serves as a "start signal" that
421 is responsible for initiation of each GnRH pulse, and by way of reciprocal connections,
422 activates other NK3R-containing KNDy cells and ultimately GnRH neurones.
423 Conceivably, decreased NKB and NK3R would lead to a diminished ability to initiate
424 GnRH pulses and hence a decrease in GnRH/LH pulse frequency. However, prenatal T
425 animals show the opposite, an increase in LH pulse frequency in gonadal-intact ewes
426 during anoestrus and the luteal phase of the oestrous cycle due to decreased
427 responsiveness to the negative feedback influence of oestradiol and progesterone,
428 respectively (36, 38, 46). However, in addition to NKB, dynorphin peptide expression
429 is also reduced in KNDy cells of prenatal T sheep (38). Evidence in ruminants supports
430 the role of dynorphin in KNDy cells as a "stop" signal, terminating each GnRH/LH pulse
431 (19). Hence it is possible that reductions in dynorphin signaling compensate for that
432 of NKB and NK3R, rendering the KNDy network less responsive to the negative
433 feedback influence of oestradiol and progesterone in prenatal T animals. Nonetheless,
434 although KNDy cells are known to be potential targets for direct actions of oestradiol
435 and progesterone (16), we do not know whether these gonadal hormones inhibit
436 GnRH/LH pulse frequency by acting directly on KNDy cells or indirectly via afferents
437 from other cells. Evidence from KNDy cell-ablated rats suggests that while KNDy cells
438 participate in the negative feedback influence of oestradiol on LH, other non-KNDy
439 cells and pathways may also play a role (54). Similarly, despite the importance of NKB
440 signaling on kisspeptin and hence GnRH stimulation, recent reports indicate that

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441 prenatal dihydrotestosterone treatment of peripubertal rats leads to elevated LH
442 responses to i.c.v kisspeptin administration (55). This, once again is in accordance
443 with decreased dynorphin expression rather than a reduction in NKB/NK3R signaling.

444 Another possibility is that decreased NKB/NK3R in prenatal T sheep is related to deficits
445 in the amplitude of the LH surge as seen in these animals (33, 45). KNDy cells in the
446 sheep express Fos, a marker of neuronal activation, during the preovulatory LH surge
447 (22, 27, 28); i.c.v. injections of senktide, which elicit a surge-like elevation in LH, also
448 induce Fos in ARC KNDy cells (23). While kisspeptin mRNA and peptide expression in
449 KNDy cells is increased during the late follicular phase in the ewe (24, 25), an
450 oestradiol stimulus that induced an LH surge was unable to increase in mRNA levels
451 for NKB in the ARC (52). In addition, although NKB agonist injections locally into the
452 POA and RCh, like i.c.v. injections result in a prolonged surge-like elevation of LH (see
453 below), senktide injections in the ARC cause only a modest increase in LH (23)
454 consistent with the role of NK3R in pulse generation in this region. Nonetheless, the
455 possibility that NK3R signaling in KNDy cells plays a role in the generation of the LH
456 surge in the ewe needs to be tested directly by NK3R antagonist injections directly into
457 the ARC in follicular phase ewes. In contrast to the reduction in NK3R-IR cells we
458 observed in the ARC, no changes were seen in the POA or in any other hypothalamic
459 nuclei analyzed in this study. We were particularly surprised by the absence of any
460 changes in the POA and RCh since senktide microinjections into either region is able
461 to elicit a surge-like pattern of LH release (23) similar to that seen after i.c.v. injections
462 of this agonist (9). Microimplants containing NKB antagonist (SB222200) into the RCh
463 but not the POA reduce the amplitude of the LH surge by 40%, suggesting that NKB
464 release in the RCh during the follicular phase is physiologically important to the
465 generation of the LH surge (23). Since i.c.v. injections of kisspeptin antagonists in
466 follicular phase ewes only reduce surge amplitude by 50% (25), it is tempting to
467 speculate that NKB and kisspeptin act synergistically to elevate LH release during the
468 surge. Interestingly, tract tracing data demonstrate that KNDy neurones receive direct
469 input from neurones in the Rch (56) and administration of senktide into the RCh
470 induces c-Fos expression in the ARC population (23). Taken together, these findings
471 suggest that NKB signaling in the RCh plays a role in the preovulatory LH surge, and
472 that the effect of NKB in the RCh is likely mediated, at least in part, by projections to
473 ARC KNDy neurones. We are currently investigating the existence of reciprocal
474 connection from the ARC to the Rch, which could constitute a potential pathway via
475 which NKB/NK3R and kisspeptin/Kiss1r signaling are involved in the GnRH surge

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476   mechanism. If NKB/NK3R in KNDy cells contribute to this mechanism, then
477   administration of the NKB agonist, senktide, should, at least partially, reverse the
478   defects in GnRH surge amplitude seen in prenatal T-treated ewes.

479   In the present study, as a marker for KNDy cells we used kisspeptin immunoreactivity.
480   Although the presence of KNDy cells has been confirmed in various species (12, 17),
481   it must be noted that single-labeled populations of NKB and/or kisspeptin cells have
482   been documented in male humans (18) and mice (15, 57). Therefore, we cannot
483   exclude the possibility that we are overseeing potential changes in co-localization of
484   NK3R and NKB-only cells. However, since previous work has shown that prenatal T
485   decreases NKB and dynorphin, but not kisspeptin expression (38), we chose kisspeptin
486   as a marker for KNDy cells, as this peptide would not be affected by the treatment
487   itself.

488   Although the above evidence supports a central role for NKB in regulating GnRH
489   secretion, this influence in the ewe has been thought to be largely indirect, based on
490   the complete absence of NK3R-IR colocalization in ovine GnRH cells (14). Instead, the
491   stimulatory influence of NKB in KNDy cells on GnRH secretion is thought to be
492   conveyed by kisspeptin as an output signal, acting upon either GnRH cell bodies or
493   terminals (16). Evidence for this upstream site of action has come from studies in
494   which kisspeptin antagonists have been shown to block the stimulatory effects of NKB
495   or senktide (58), as well as studies in which desensitization of the kisspeptin receptor
496   blocks the stimulatory effect of senktide in monkeys (59), and the absence of the
497   stimulatory effect of senktide in Kiss1r KO mice (60). The current working hypothesis
498   of the mechanisms by which NKB acts as a stimulatory "start" signal in the generation
499   of GnRH pulses in the ewe, posits this action occurring via reciprocal KNDy-KNDy
500   inputs at the level of KNDy cell bodies. Our observation in the present study of NK3R-
501   IR localization in terminals that are presynaptic to GnRH cell bodies suggest another
502   possibility – that NKB release by KNDy terminals acts in an autoregulatory manner
503   upon the same terminals contributing to enhanced release of kisspeptin. However,
504   since we did not co-localise NK3R with KNDy peptides in inputs contacting GnRH
505   neurones, we cannot conclude that the presynaptic NK3R inputs we observed arose
506   from KNDy cells and indeed may have originated from any of the number of other
507   NK3R-IR cell populations. For example, the effects of senktide injections in the POA on
508   LH secretion (23) may be mediated either by actions on NK3R-containing cell bodies
509   in that region, or by presynaptic NK3R in contact with POA GnRH neurones. Since we

510 found very little colocalization (approximately 5%) of NK3R within kisspeptin cells of
511 the POA, it is possible that senktide effects on LH secretion from injections into this
512 area are independent of kisspeptin, and mediated instead by other
513 transmitters/peptides. It is noteworthy that the effects of senktide on GnRH release in
514 tissue slices of the mouse median eminence are also independent of kisspeptin (61),
515 and since GnRH neurones in the mouse (15) like the sheep (14) lack NK3R, it is
516 possible that the effect of senktide in the median eminence is also mediated by
517 presynaptic actions of NKB, in this case via axo-axonic contacts. The possibility of
518 presynaptic actions of NKB is supported by evidence in other systems and species, for
519 example, in the rat striatum, in which tachykinins presynaptically stimulate the release
520 of dopamine (62). Finally, we would note that the observations reported here are
521 based on control animals; the possibility that changes in presynaptic localization of
522 NK3R are present in prenatal T female sheep and contribute to reproductive
523 neuroendocrine defects remains to be examined.

524 In summary, the decreases in NK3R we observed in the ARC of prenatal T-treated
525 ewes complement previous observations of decreases in NKB and dynorphin peptides
526 in KNDy cells (38), and suggest that the combined reduction in ligand and receptor
527 components of NKB/NK3R signaling may contribute to alterations in the control of
528 pulsatile or surge modes of GnRH/LH secretion.   The constellation of adult
529 reproductive dysfunction, as well as metabolic defects, in prenatal T-treated ewes is
530 very similar to that observed in women with polycystic ovarian syndrome (PCOS) (34)
531 suggesting that the prenatal T ewe may serve as a model for this disease (33). KNDy
532 cells are present in the human female infundibular nucleus (equivalent to the ARC in
533 ewes) and show morphological changes with loss of steroid feedback regulation of
534 GnRH/LH (63, 64). For example, in the infundibular nucleus of postmenopausal
535 women, NKB gene expression is elevated due to reduced oestrogen negative feedback
536 (65). Thus, we would speculate that alterations in NKB/NK3R signaling may be, at
537 least in part, responsible for the ovulatory defects observed in patients with PCOS.
538 The prenatal T-treated ewe could serve as an important translational model to test
539 this hypothesis, with regard to the feedback control of GnRH/LH pulses as well as the
540 generation of the preovulatory LH surge.

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Figure Legends

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799 **Figure 1.** Schematic drawings of coronal sections through the ovine POA and

800 hypothalamus, depicting the distribution of NK3R-IR cells. Each solid circle represents

801 approximately 10 NK3R-IR cells. Abbreviations; (A) BNST: Bed nucleus of stria

802 terminalis; GP: globus pallidus; ac: Anterior commissure; POA: preoptic area; SON:

803 superior optic nucleus; OVLT: organum vasculosum of lamina terminalis; SI:

804 substantia innominata; OC: optic chiasm;   (B) fx: fornix; PVN: paraventricular

805 nucleus; 3V: 3rd ventricle; IC: internal capsule; AHA: anterior hypothalamic area; OT:

806 optic tract; LHA: lateral hypothalamic area; (C) RCh: retrochiasmatic area; (D) ZI:

807 zona incerta ; mt: mammillary tract; mARC: middle arcuate; (E) CP: cerebral

808 peduncle; PMv: premammillary ventricle; cARC: caudal arcuate; mr: mammillary

809 recess.

810

811 **Figure 2. (A)** Mean (± SEM) number of NK3R immunoreactive cells/hemisection in

812 POA, PVN, Rch, LHA and PMv of control (n=9) and prenatal T-treated (n=8) groups.

813 There were no statistically significant differences between control and prenatal T-

814 treated ewes in these areas. **(B)** Mean (± SEM) number of NK3R immunoreactive

815 cells/hemisection in the middle and caudal ARC from control (n=9) and prenatal T-

816 treated (n=8) groups. * indicates statistically significant differences within each

817 subdivision compared to controls (P<0.05). **(C-F)** Representative images showing

818 examples of NK3R-IR cells (arrows) in the ARC of control (C, mARC; D, cARC) and

819 prenatal T-treated ewes (E; mARC; F, cARC). Scale bar = 50 μm.

820

821 **Figure 3. (A-F):** Confocal images (1 μm optical sections) showing dual-label

822 immunofluorescent detection of NK3R-IR and kisspeptin-IR in the middle ARC of

823 control (A-C) and prenatal T-treated ewes (D-F). Arrows indicate examples of dual-

824 labelled neurones. Scale bar = 20 μm (63 x). **(G)** Mean (± SEM) number of single-

825 labelled NK3R, single-labelled kisspeptin (Kiss), dual-labelled NK3R and kisspeptin

826 (NK3R/Kiss), and total kisspeptin and NK3R-IR neurones in the ARC of control (n=9)

827 and prenatal T-treated (n=8) ewes. **(H)** Mean (± SEM) percentage of kisspeptin cells

828 co-localizing NK3R (%Kiss/NK3R; left) and percentage of NK3R cells co-localizing

829 kisspeptin (%NK3R/Kiss; right) in the ARC of control (n=9) and prenatal T-treated

830 (n=8) groups. * indicates statistically significant difference compared to controls

831 (P<0.05).

832

833   **Figure 4.** Triple-label detection of NK3R, GnRH and synaptophysin (Syn) in a 1μm

834   confocal optical section, demonstrating the presence of presynaptic NK3R-IR terminals

835   in contact with GnRH neurones in the MBH. Scale bar = 10 μm.

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837

838   **Table 1. Presynaptic NK3R terminals in contact with GnRH somas.** Mean (±

839   SEM) percentage of GnRH neurones receiving one or more NK3R-IR contact, mean

840   number of NK3R-IR contacts per GnRH soma, and mean number of NK3R-IR contacts

841   per 10 μm cell surface, for GnRH cells in the POA and MBH of control ewes (n=5).

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Control (n=9)
Prenatal T (n=8)

VN   Rch   LHA   PMv

Control (n=9)
Prenatal T (n=8)

*

*

ARC   Caudal ARC

C

mARC

D

cARC

NK3R   Kiss   M

Control (n=9)
Prenatal T (n=8)

Single Kiss   Dual NK3R/Kiss   Total NK3R   Total Kiss

H

% Colocalization

Control (n=9
Prenatal T (

% Kiss/NK3R