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# Expression of genes for Kisspeptin (*KISS1*), Neurokinin B (*TAC3*), Prodynorphin (*PDYN*), and gonadotropin inhibitory hormone (*RFRP*) across natural puberty in ewes

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**Abstract**

Expression of particular genes in hypothami of ewes was measured across the natural pubertal transition by in situ hybridization. The ewes were allocated to three groups ($n = 4$); prepubertal, postpubertal and postpubertally gonadectomized (GDX). Prepubertal sheep were euthanized at 20 weeks of age and postpubertal animals at 32 weeks. GDX sheep were also euthanized at 32 weeks, 1 week after surgery. Expression of *KISS1*, *TAC3, PDYN* in the arcuate nucleus (ARC), *RFRP* in the dorsomedial hypothalamus and *GNRH1* in the preoptic area was quantified on a cellular basis. *KISS1R* expression by *GNRH1* cells was quantified by double-label in situ hybridization. Across puberty, detectable *KISS1* cell number increased in the caudal ARC and whilst *PDYN* cell numbers were low, numbers increased in the rostral ARC. *TAC3* expression did not change but *RFRP* expression/cell was reduced across puberty. There was no change across puberty in the number of *GNRH1* cells that expressed the kisspeptin receptor (*KISS1R*). GDX shortly after puberty did not increase expression of any of the genes of interest. We conclude that *KISS1* expression in the ARC increases during puberty in ewes and this may be a causative factor in the pubertal activation of the reproductive axis. A reduction in expression of *RFRP* may be a factor in the onset of puberty, removing negative tone on GNRH1 cells. The lack of changes in expression of genes following GDX suggest that the effects of gonadal hormones may differ in young and mature animals.

**KEYWORDS**

gonadotropin releasing hormone, gonadotropins, hypothalamus

## 1 | INTRODUCTION

Puberty is typified by an increase in the secretion of gonadotropin releasing hormone (GnRH) which drives an increase in gonadotropins secretion from the pituitary gland (Clarke & Pompolo, 2005; Ojeda, Roth, et al., 2006), leading to activation of the gonads. GnRH neurons are controlled by a number of interactive neuronal pathways which are regulated by internal signals and external cues (Clarke, 2015; Clarke & Arbabi, 2015; Clarke, Campbell, Smith, Prevot, & Wray,

2011; Ojeda, Lomniczi, et al., 2006; Tena-Sempere, 2012; Terasawa & Fernandez, 2001), many of which may be involved in the process of puberty. Although much is known about neuronal systems that regulate GnRH secretion, the neurochemical basis of integrated control of puberty remains only partially understood. In higher primates, there is a "brake" on the secretion of GnRH and gonadotropins prior to puberty that is not due to feedback effects of steroids from the gonads (Plant, 2015; Plant & Shahab, 2002). In species such as the sheep, however, pulsatile GnRH secretion and gonadotropin secretion is apparent in the prepubertal period (I'Anson et al., 2000) but is held in check by an enhanced negative feedback action of estrogen (Foster & Ryan, 1979).

Since realization that kisspeptin is a major regulator of GnRH secretion (de Roux et al., 2003; Gottsch et al., 2004; Irwig et al., 2004; Messager et al., 2005; Seminara et al., 2003), its function was shown to be mandatory for pubertal transition (de Roux et al., 2003; Seminara et al., 2003). Nevertheless, the question as to whether altered function of kisspeptin is the primary and essential driver of the increase in GnRH that occurs at the time of puberty remains a matter of debate. There are two populations of kisspeptin neurons in hypothalamus which are differentially regulated by sex steroids. One population in the anteroventral periventricular nucleus (AVPV) in rodent or preoptic area (POA) in species such as sheep is involved in the positive feedback action of estrogen that causes the GnRH/luteinizing hormone (LH) surge in females (Hoffman, Le, Franceschini, Caraty, & Advis, 2011; Robertson, Clifton, Iglesia, Steiner, & Kauffman, 2009; Smith, Li, Pereira, & Clarke, 2009; Smith, Popa, Clifton, Hoffman, & Steiner, 2006). A second group of kisspeptin cells is located in the arcuate nucleus (ARC) and relays the negative feedback effects of sex steroid on GnRH secretion in both sexes (Franceschini et al., 2006; Smith, Cunningham, Rissman, Clifton, & Steiner, 2005). In sheep, kisspeptin neurons in caudal ARC also initiate the positive feedback effect of estrogen on GnRH secretion (Estrada, Clay, Pompolo, Smith, & Clarke, 2006; Smith, 2008; Smith et al., 2009), which is potentiated by activation of the preoptic kisspeptin neurons at the time of the surge (Hoffman et al., 2011).

Mutations in either the kisspeptin gene (*KISS1*) or the kisspeptin receptor (*KISS1R*) in humans and gene knockout in mice causes pubertal failure (d'Anglemont de Tassigny & Colledge, 2010; Dungan Lemko & Elias, 2012; de Roux et al., 2003; Seminara et al., 2003; Topaloglu et al., 2012). Nevertheless, a range of other gene mutations can also cause failure of puberty (Silveira, Trarbach, & Latronico, 2010). Developmental changes in *KISS1* expression have been described in many species, with variable patterns within and between species. In mice and rats, *KISS1* is expressed in the ARC before birth but some studies did not detect significant changes in expression in the ARC at the time of puberty in either species (Gill et al., 2010; Han et al., 2005; Navarro et al., 2012). Others (Molnar et al., 2016) have reported an increase in expression of *KISS1* in male mice at the time of puberty. Increased *KISS1* expression in the early stages of puberty has been observed in both male and female monkeys and rats (Bentsen et al., 2010; Shahab et al., 2005; Takase et al., 2009). No change in *KISS1* expression was observed in the ARC of female pigs across puberty (Ieda et al., 2014).

In female sheep, the enhanced negative feedback of estradiol-17β (E2) in the prepubertal period is lost at puberty onset (Foster & Ryan, 1979). One study showed that, in ovariectomized (OVX)-E2-treated ewes, escape from the estrogenic "clamp" occurs at 35 weeks, typified by a marked increase in pulsatile LH secretion (Redmond et al., 2011). There was, however, no change in *KISS1* expression in the ARC of these animals, although an increase was observed in the POA by 30 weeks. In another study, immunohistochemistry showed the presence of more kisspeptin cells in ARC in adult ewes (>9 month) than in prepubertal ewes (5–6 months) (Nestor et al., 2012), consistent with the notion that kisspeptin may facilitate if not initiate puberty. No studies have been performed on gonad-intact ewes across the pubertal transition to determine any potential change in *KISS1* gene expression.

Kisspeptin cells of the ARC also express *PDYN* and *TAC3*, the genes encoding dynorphin (DYN) and neurokinin B (NKB) (Goodman et al., 2007), leading to nomenclature of "KNDY" cells (Lehman, Coolen, & Goodman, 2010; Maeda et al., 2010; Navarro et al., 2009; Rance, Krajewski, Smith, Cholanian, & Dacks, 2010). NKB may act in an "autocrine" manner to stimulate kisspeptin secretion, whereas DYN inhibits KNDY cell function (Goodman, Coolen, & Lehman, 2014; Grachev et al., 2014; Sakamoto et al., 2012). Mutations in *TAC3* or the NKB receptor cause hypogonadotropic hypogonadism in humans (Gianetti et al., 2010; Topaloglu et al., 2009). NKB agonists can stimulate LH secretion prior to puberty in rats (Ruiz-Pino et al., 2012), ewes (Nestor et al., 2012) and juvenile primates (Ramaswamy et al., 2010). In rats, developmental changes of *TAC3* expression show a steady increase from birth until the juvenile stage, but there is little change in the transition through puberty (Navarro et al., 2012). Most recently, it has been proposed that NKB/kisspeptin interaction is enhanced during the pubertal transition in female, but not male rhesus monkeys (Garcia, Keen, Kenealy, Seminara, & Terasawa, 2018). Immunoreactive NKB cell numbers were seen to be similar in prepubertal (5–6 months) and postpubertal ewes (>9 months) (Nestor et al., 2012).

DYN has an inhibitory effect on GnRH neurons as do other opioid peptides (Goodman et al., 2004; Navarro et al., 2009; Yen, Quigley, Reid, Ropert, & Cetel, 1985). This may be due to its action on kisspeptin cells or directly upon GnRH cells, which express the relevant receptor (Weems et al., 2016). In sheep, DYN neurons in the ARC appear to mediate the negative feedback effect of progesterone on GnRH/LH pulse secretion (Goodman et al., 2004, 2011; Goodman, Parfitt, Evans,

Dahl, & Karsch, 1995). Thus, the numbers of DYN neurons are reduced in OVX ewes and restored by progesterone treatment (Foradori, Goodman, Adams, Valent, & Lehman, 2005). This is consistent withresults in human studies (Rometo & Rance, 2008), but not with data on manipulation by sex steroids in rats and nonhuman primates (Eghlidi, Haley, Noriega, Kohama, & Urbanski, 2010; Navarro et al., 2009). There are few studies of the role that DYN might play during puberty, but administration of a kappa receptor antagonist did advance puberty in female rats (Nakahara et al., 2013).

Gonadotropin inhibitory hormone (GnIH), also known as RF-amide-related peptide (RFRP) is a negative regulator of GnRH/gonadotropin function in various species, including sheep (Clarke et al., 2008, 2011, 2012; Clarke & Parkington, 2014; Clarke & Smith, 2010; Gibson et al., 2008; Johnson, Tsutsui, & Fraley, 2007; Kadokawa et al., 2009; Kriegsfeld et al., 2006; Murakami et al., 2008; Tsutsui et al., 2000). Administration of GnIH antisense oligonucleotide to juvenile male rats increased plasma LH levels and testicular weight (Johnson & Fraley, 2008) and RFRP receptor knock-out mice have elevated plasma LH levels (Leon et al., 2014). In both studies, animals were fertile and had normal pubertal onset, providing no strong evidence that a change in GnIH function is important in reproductive development. On the other hand, *RFRP* expression changes with development in rodents, with a postnatal rise, a peak in expression at the time of puberty, and a decline in adulthood in both rats and mice (Iwasa et al., 2012; Poling, Kim, Dhamija, & Kauffman, 2012; Quennell, Rizwan, Relf, & Anderson, 2010). Strangely, RFRP-3 stimulates LH secretion in male mice but is inhibitory in females (Ancel, Inglis, & Anderson, 2017). How this relates to control of the onset of puberty in either sex is not clear.

This study aimed to clarify the role of kisspeptin, NKB, DYN in the ARC, and RFRP in the DMH across puberty in ewes by measuring expression of the genes for the peptides. In addition, we examined if removal of sex steroid feedback by GDX shortly after puberty indicated whether the feedback loops evident in adult animals are fully developed at this time.

## 2 | MATERIAL AND METHODS

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

### 2.1 | Animals and experimental design

Corriedale ewes were born in September in the Southern Hemisphere and were allocated into three groups ($n = 4$), being prepubertal, postpubertal and postpubertally gonadectomized (GDX). Prepubertal sheep were euthanized at 20 weeks (February). The estrous cycles of postpubertal intact ewes were synchronized by an injection (i.m.) of a synthetic prostaglandin (Estrumate, 125 μg; Pitman-Moore) and were euthanized 10 days later during luteal phase at 32 weeks of age (April). The ovaries were examined and corpora lutea and large follicles were observed in all postpubertal ewes. Gonadectomies were performed one week prior to euthanizing at 32 weeks. Prior to perfusion, three jugular venous blood samples were collected and LH was measured as described previously (Lee et al., 1976). Body weights and plasma LH levels are shown in Table 1.

### 2.2 | Tissue processing

The sheep were euthanized with an IV overdose of sodium pentobarbital (Lethabarb; Virbarc) and the brains were perfused with paraformaldehyde as described previously (Smith et al., 2009). Hypothalami were dissected out of the brains and postfixed for 24 hr at 4°C, and then placed in phosphate buffer containing 30% sucrose for 7 days. The hypothalamic blocks were frozen and stored at −20°C. Cryostat sections were cut at 30 μm and stored in cryoprotectant solution at −20°C.

### 2.3 | In situ hybridization

Antisense riboprobes for *KISS1*, *TAC3, PDYN*, *RFRP, GNRH1,* and *KISS1R* were synthesized with $SP^6$ or $T^7$ transcription kits (Ambion), using corresponding DNA fragments as templates. A 262bp antisense riboprobe for *TAC3* (Genbank accession number XM_004006562.1, bases 31–292) was prepared as described previously (Li, Millar, Clarke, & Smith, 2015). For *PDYN*, a 200 bp fragment of the bovine gene in pCRII (Genbank accession number

**TABLE 1** Mean (±*SEM*) body weights and plasma LH concentrations of ewes prior to and postpuberty and after GDX

| | Prepuberty | Postpuberty | Postpuberty GDX |
|---|---|---|---|
| Bodyweight (kg) | 27.25 ± 0.66 | 28.38 ± 0.80 | 30.88 ± 0.43 |
| LH (ng/ml) | 0.18 ± 0.09 | 0.33 ± 0.07 | 1.1 ± 0.41 |

U58500.1) was provided as a gift by Dr. Hong Jiang, University of Missouri); the use of this probe in sheep has been reported previously (Iqbal, Henry, Pompolo, Rao, & Clarke, 2003). The *KISS1*-specific template spanned bases 1–357 of the partial ovine cDNA sequence (GenBank accession no. DQ059506) and a 460 bp cDNA sequence of the ovine *RFRP* (bases 43–502, GenBank accession no.NM_001127268) was cloned as previously described (Clarke et al., 2008). The *KISS1R*-specific template spanned bases 6–636 of ovine cDNA sequence (GenBank accession no. EU272411). The *GNRH1*-specific template spanned bases 18–169 of the ovine partial cDNA sequence (GenBank accession No. U02517).

In situ hybridization was performed as described previously (Simmons, Arriza, & Swanson, 1989) using $^{35}$S-labeled probes. For each sheep and each gene, three sections were selected for analysis. ARC sections were selected to represent the rostral, middle, and caudal regions. The caudal section was 150–300 μm from mammillary recess of the third ventricle and the middle and rostral sections are 600 μm apart. For sections used in *RFRP* detection, rostrocaudal sections of PVN/DMH were chosen: the caudal section was 300–450 μm from mammillary recess of the third ventricle and the middle and rostral sections are 600 μm apart. These were mounted onto SuperFrost plus slides and air-dried overnight. Following 0.001% proteinase K treatment for 30 min at 37°C, sections were acetylated with 0.0025% acetic anhydride in 0.1 M TEA for 10 min. After rinsing in 2 × SSC, the sections were dehydrated through ascending series of ethanol, delipidated in chloroform, rinsed in absolute ethanol, and air-dried. The hybridization solutions containing $^{35}$S labeled antisense probe ($5 \times 10^6$ cpm/ml) in a cocktail solution of 50% formamide, 5 × SSC, pH 7.0, 250 μg/ml herring sperm DNA, 100 μg/ml yeast tRNA, 5% dextran sulfate, 1× Denhardt's solution, 0.1% Tween-20 was applied to sections and hybridized overnight at 53°C. After hybridization, sections were washed in decreasing concentrations of SSC, dehydrated, and coated with emulsion (Ilford Imaging). Exposure was 1 week in the dark at 4°C.

Double label in situ hybridization using DIG-labeled *GNRH1* and $^{35}$S-labeled *KISS1R* riboprobes was performed as described previously (Li, Goodchild, Seyedabadi, & Pilowsky, 2005; Li, Rao, Pereira, Clarke, & Smith, 2011; Smith et al., 2011). Rostral, medial, and caudal regions were hybridized with the DIG-*GnRH1* riboprobe and the $^{35}$S-labeled *KISS1R* riboprobes ($5 \times 10^6$ cpm/ml) at 53°C overnight. After posthybridization washes with descending concentrations of citrate acid and NaCl (SSC), sections were rinsed twice in Tris-buffered saline (TBS) (0.1 M Tris–HCl, 0.9% NaCl, pH 7.4). The *GNRH1* expressing neurons were revealed with an alkaline phosphatase conjugated goat anti-digoxigenin antibody (dilution 1:1,000; Roche) and a colorimetric solution of nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate salts (Roche). The $^{35}$S signal for *KISS1R* was revealed on GnRH neurons, as silver grain staining. The sections were coated with 3% parlodion in isoamyl acetate, dried, dipped in photographic emulsion (Ilford Imaging), and left at 4°C for 5 weeks. Grain-counting software (Image-Pro plus; Media Cybernetics) was used to count the number of *KISS1R* mRNA silver grains over each GnRH cell under darkfield illumination. The signal-to-noise ratio was set at the 3× background.

For each gene, a sense probe, using the same template as antisense, was used as a negative control to assess nonspecific hybridization.

### 2.4 | Microscopy

Image analysis was carried out using randomly coded slides under dark-field illumination with software designed to count the total number of cells and the number of silver grains per cell (ImagePro plus, Media Cybernetics, Inc.). Cells were counted when silver grain density was three times greater than the background. Data are expressed as the mean number of identifiable cells/section and the mean number of silver grains/cell (a semiquantitative index of mRNA expression/cell). Densitometry (expression/cell) for *PDYN* was not performed as there were very few cells detected in the ARC.

### 2.5 | LH Radioimmunoassay

Plasma LH concentrations were measured in duplicate, using the method of Lee et al. (1976). Assay sensitivity was 0.1 ng/ml and the intra-assay coefficient of variation (CV) was less than 10% over the range of 0.6–15 ng/ml.

### 2.6 | Statistical analysis

All grouped data are presented as the means (±*SEM*). Statistical analyses were conducted after checking for heterogeneity of variance, by one-way ANOVA. Differences were considered significant at $p < .05$.

## 3 | RESULTS

Plasma LH levels increased from 0.33 ± 0.07 ng/ml to 1.1 ± 0.41 ng/ml following GDX ($p < .05$) (Table 1).

Examples of the in situ hybridization signal for *KISS1*, *TAC3, PDYN*, and *RFRP* are shown in Figure 1. These images indicate high signal-to-noise and clear silver grain concentration over the relevant cells. For all probes, no signal was observed after the application of radioactive-labeled sense probes.

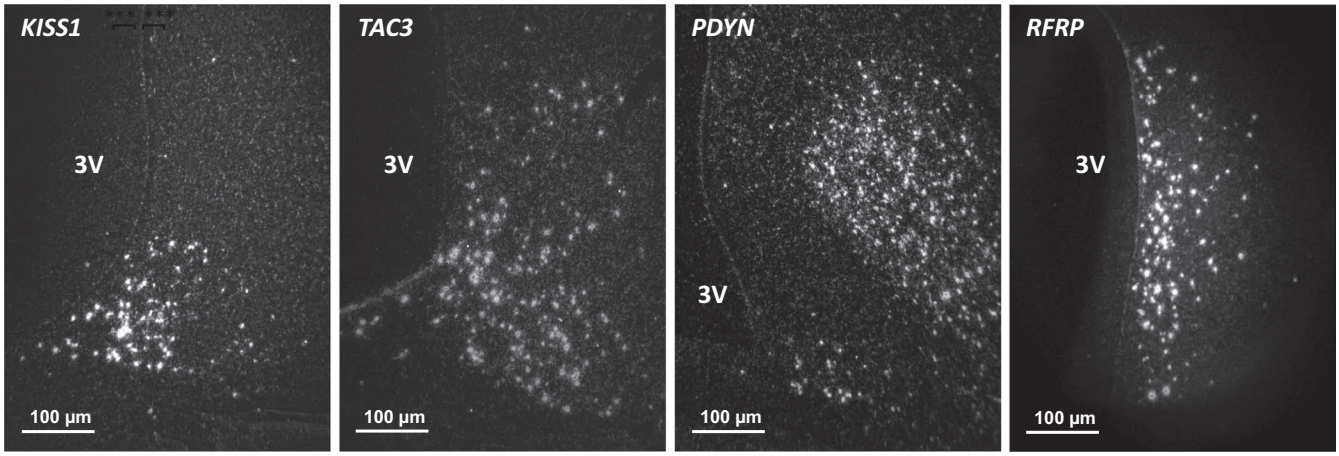


**FIGURE 1** Representative microphotographs of the in situ hybridization signal for *KISS1*, *TAC3*, *PDYN* in arcuate nucleus (ARC) and *RFRP* in the dorsomedial hypothalamus

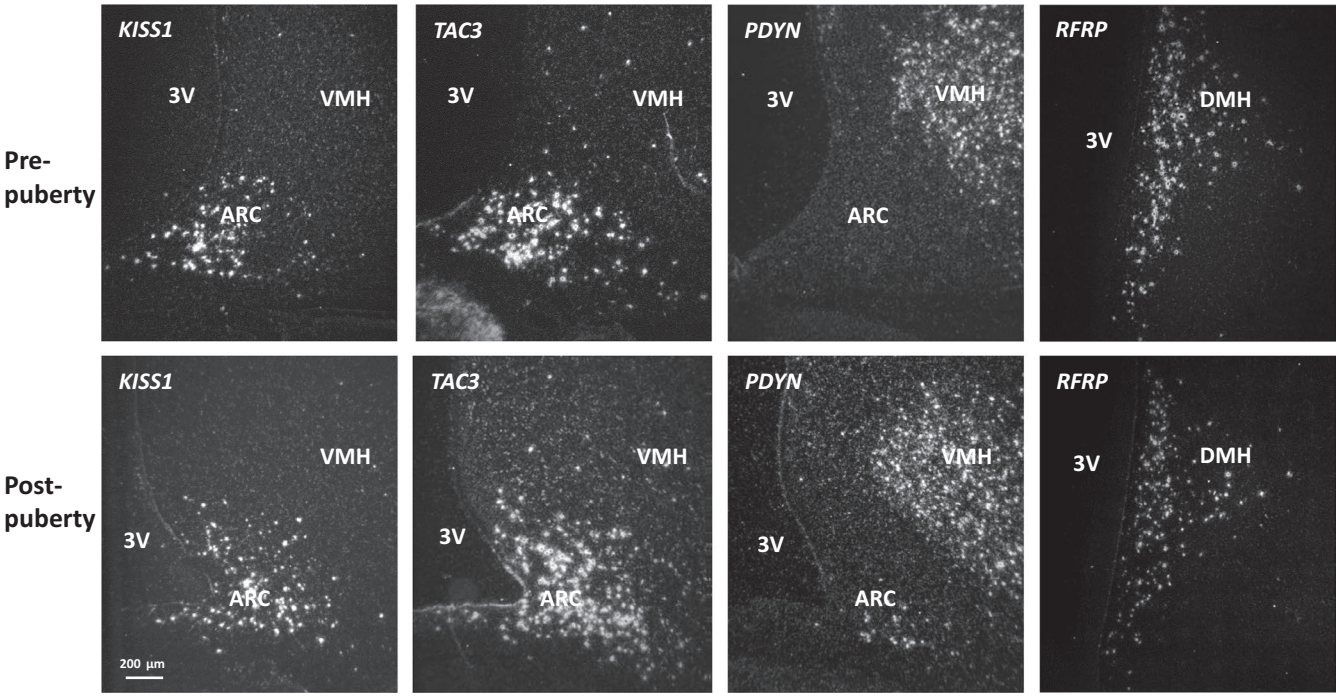


**FIGURE 2** Examples of *KISS1*, *TAC3*, *PDYN* gene expression at the same level in arcuate nucleus and *RFRP* in the dorsalmedial nucleus prior to and after puberty in ewes. Note the fewer number of *PDYN* expressing cells. ARC, arcuate nucleus; VMH, ventromedial hypothalamus; DMH, dorsalmedial hypothalamus; 3V, third ventricle

### 3.1 | *KISS1*, *TAC3*, *PDYN*, and *RFRP* expression

*KISS1* cell number in the caudal ARC was higher ($p < .05$) in postpubertal ewes than in prepubertal ewes (Figures 2 and 3a), with no change in expression/cell (Figure 3a). GDX did not change *KISS1* expression in postpubertal females (Figure 3a).

The number of TAC3 cells and the expression of *TAC3*/cell was similar in prepubertal, postpubertal intact and GDX ewes (Figures 2 and 3b).

*PDYN* mRNA was highly expressed in supraoptic nucleus (SOR), dorsal medial hypothalamus (DMH) (data not shown), and the ventromedial hypothalamus (Figure 2). *PDYN* expression was low in the ARC, with the number of cells being less than observed for *KISS1* and *TAC3* cells (Figures 2 and 3). *PDYN* cell number increased ($p < .01$) in the rostral ARC across puberty and GDX reduced cell numbers (Figure 3c, $p < .001$).

The number of *RFRP*-expressing cells was similar in prepubertal, postpubertal intact, and GDX females, but *RFRP* expression/cell was lower ($p < .01$) after puberty at all levels

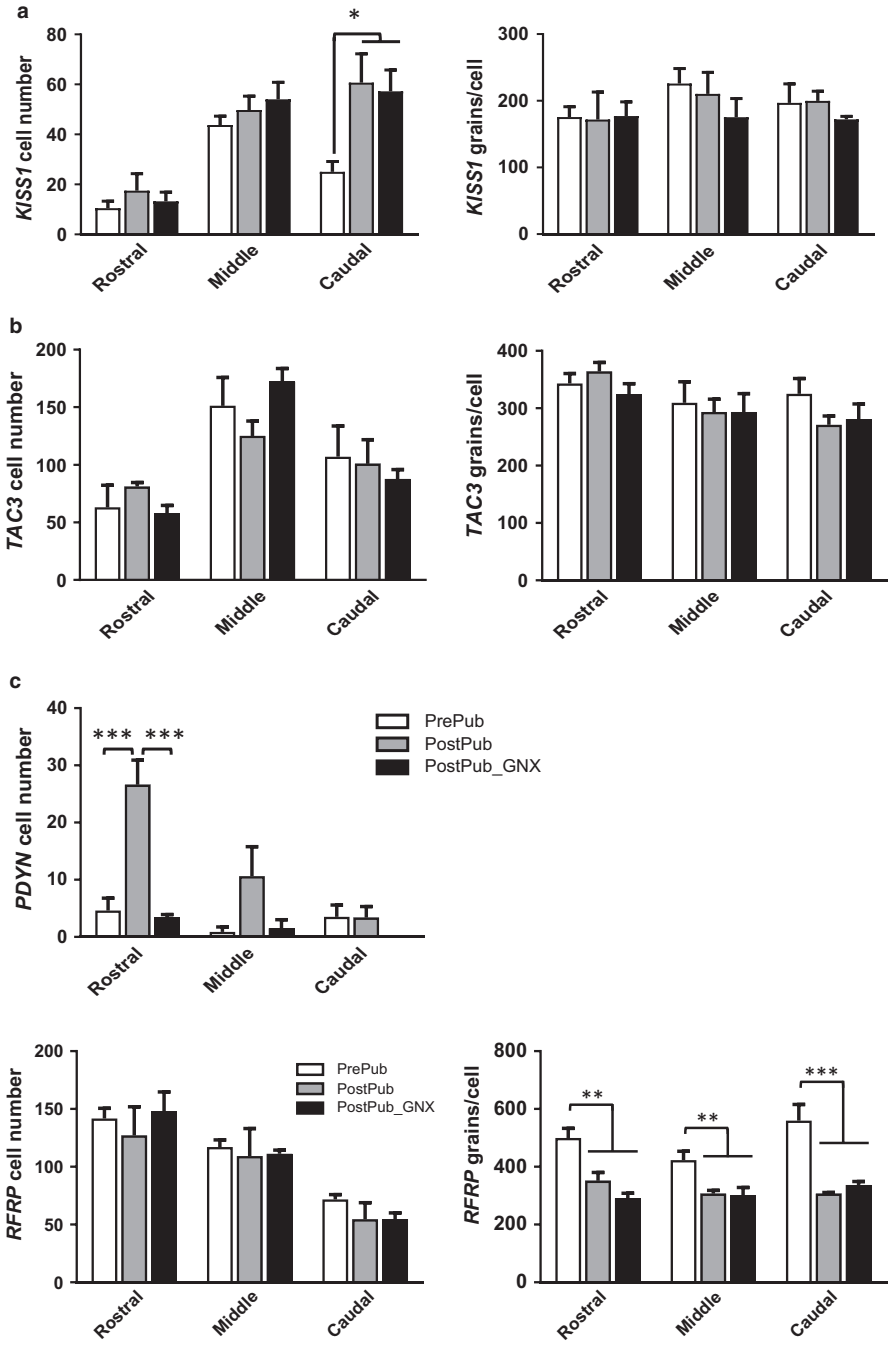


**FIGURE 3** Mean (±*SEM*) expression of *KISS1* (a), *TAC3* (b), and *PDYN* (c) in the ARC of ewes prior to and postpuberty and following GDX. Panels on the left show cell number and those on the right show expression/cell (silver grains/cell) in rostral mid and caudal sections of the ARC. $*p < .05$, $***p < .001$

**FIGURE 4** Mean (±*SEM*) *RFRP* gene expression in the DMH of ewes prior to and postpuberty and following GDX. Panels on the left show cell number and those on the right show expression/cell (silver grains/cell) in rostral mid and caudal sections of the dorsomedial nucleus. $**p < .01$, $***p < .001$

of the dorsomedial nucleus (Figure 4). The level of expression after puberty was not affected by GDX (Figure 4).

### 3.2 | *KISS1R* expression in *GNRH1* cells

The number of *GNRH1* expressing neurons was similar in pre and postpubertal sheep (intact or GDX (data not shown). Here $^{35}S$ labeled *KISS1R* expression was distinctively visible on digoxigenin-labeled GnRH neurons (Figure 5a). *KISS1R* expression/cell and the percentage of GnRH neurons that expressed *KISS1R* were similar before and after puberty (Figure 5b).

## 4 | DISCUSSION

It is generally accepted that kisspeptin signaling is mandatory for puberty (d'Anglemont de Tassigny & Colledge, 2010; Dungan Lemko & Elias, 2012; de Roux et al., 2003; Seminara et al., 2003; Topaloglu et al., 2012), but as to whether an increase in expression of *KISS1* or *KISS1R* is seen at this time across all species is questionable. In this study, we focused on the *KISS1* cells of the ARC of the sheep, because an increase in expression.in the POA was recorded in earlier work (Redmond et al., 2011). The present data show that the number of cells expressing *KISS1* in the caudal ARC is higher shortly after puberty in female sheep. The increased number

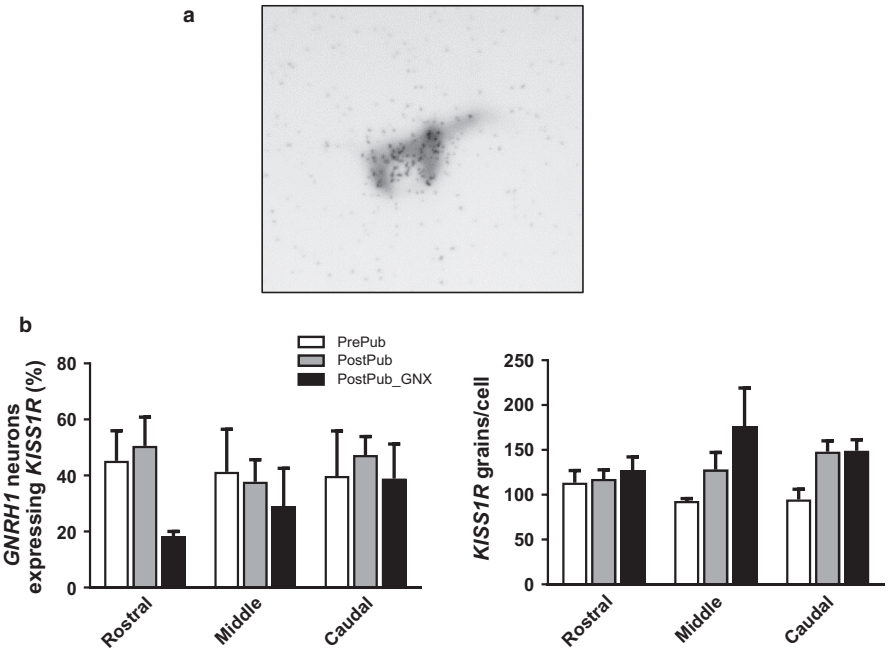


**FIGURE 5** *KISS1R* expression in *GnRH1* cells. Panel A shows co-localization of *KISS1R* (silver grains; $^{35}$S-labeled riboprobe) in a Digoxigenin-labeled *GNRH1* neuron visualized in gray-scale. Panel B shows mean (±*SEM*) % *KISSR* expression by *GNRH1* cells (left panel) and silver grain density per cell (right panel)

of *KISS1* expression in the ARC of gonad-intact ewes after puberty is consistent with the increased expression seen in the POA of OVX ewes-bearing estrogen implants (Redmond et al., 2011). The rise in *KISS1* function in the POA at the time of puberty seems most likely dependent on the action of estrogen on *KISS1* neurons in this location, based on studies in mice (Clarkson, Han, Liu, Lee, & Herbison, 2010). On the other hand, some studies report no change in expression of *KISS1* was seen across the pubertal period in female pigs or in mice and rats of either sex (see Introduction).

We found no change of expression of *KISS1* in the ARC of ewes after GDX soon after puberty, which contrasts with our earlier results obtained with mature ewes (Smith, Clay, Caraty, & Clarke, 2007). This may be because the animals of this study were euthanized one week after GDX or it could be due to there being relatively less influence of gonadal steroids on *KISS1* expression in the early postpubertal stage of development. Certainly there is overwhelming evidence that *KISS1* expression in the ARC increases with GDX of either sex in adult rodents (Irwig et al., 2004; Kauffman et al., 2007; Navarro et al., 2009; Smith, Cunningham, et al., 2005; Smith, Dungan, et al., 2005) and an increase is also seen in female primates after GDX or menopause (Kim, Jessen, Auger, & Terasawa, 2009; Rometo & Rance, 2008). Despite there being no increase in *KISS1* after GDX, the plasma levels of LH increased in both males and females. This suggests that the negative feedback effects of gonadal steroids on GnRH secretion may be mediated by neural elements other than KNDY cells. Certainly, a number of neuronal systems express estrogen receptors (Clarke & Tilbrook, 2009) and could be involved in the suppression of GnRH/LH secretion at this time. In addition, the gonadotropes express sex steroid receptors and negative feedback is effected at that level (Clarke, Cummins, Crowder, & Nett, 1989).

The percentage of *GNRH1* cells expressing *KISS1R* did not change across puberty. The lack of effect of GDX is consistent with results in male rhesus monkeys at the time of expected puberty (Shahab et al., 2005), with similar results in male mice (Molnar et al., 2016) and female rats (Adachi et al., 2007). Neither does *KISS1R* expression change in female rhesus monkeys in the transition to menopause (Kim et al., 2009). On balance, it seems most likely that the transition through puberty is associated with upregulation of kisspeptin activity rather than that of its receptor.

Regarding *TAC3* expression we found no change across puberty or after GDX, consistent with immunohistochemical data on female sheep (Nestor et al., 2012). Our data also concur with data obtained from rats (Navarro et al., 2012). Others (Gill et al., 2012) presented data from female mice and showed that *TAC3* and its receptor (*TAC3R*) were most likely markers of pubertal activation but were not triggers for puberty while data from male mice showed no change in *TAC3R* (Molnar et al., 2016). Overall, there seems to be no significant role for NKB in the initiation of puberty in either sex, even though mutations in *TAC3* and *TAC3R* genes lead to reproductive failure (Silveira et al., 2010).

Around the time of puberty, the number of detectable *PDYN* neurons was lower than for *KISS1* and *TAC3* neurons, which is consistent with other data obtained in female sheep showing very few *PDYN* cells in the prepubertal ewe (Lopez et al., 2016). The striking difference in the number of neurons expressing *TAC3*, *KISS1*, and *PDYN* at this stage of development is interesting, considering that the three peptides co-localized in the KNDy neurons in the adult sheep (Goodman et al., 2007). This suggests that the neuroendocrine axis governing reproductive function is different in young and adult animals and, as suggested earlier (Lopez

et al., 2016), a rise in progesterone levels may be necessary for induction of *PDYN* expression, perhaps explaining why the number of cells increased markedly in the rostral ARC, following puberty. In spite of the low number of cells, we showed that GDX reduced the number of *PDYN* cells. These data are consistent with a fundamental role for this peptide in the negative control of pulsatile GnRH secretion. The low cell number precluded meaningful quantification of the level of expression/cell.

A steady increase in GnIH levels in the hypothalami of male and female rats has been seen across development (Iwasa et al., 2012). Likewise, Poling et al (Poling & Kauffman, 2012) showed a steady increase of GnIH levels in a subpopulation of GnIH neurons in mice of both sexes, whereas Quennell et al (Quennell et al., 2010) showed that *RFRP* expression in the dorsomedial nucleus of male mice peaked at 4 weeks. Nevertheless, this maximal level of expression at 4 weeks was followed by a progressive decline up to 8 weeks, although this was not statistically significant. In another study of mice, a marked increase in *RFRP* cell number was observed at 3 weeks of age in males, with a progressive decline occurring between 7 and 13 weeks of age (Sethi, Tsutsui, & Chaturvedi, 2010). We observed a reduction in the level of expression of RFRP/cell in females after puberty which is not consistent with the aforementioned data obtained in rats and mice but is consistent with there being a release from inhibitory influence on GnRH cells at the time of puberty. When considering the role of GnIH in control of reproduction, it should be noted that this peptide is stimulatory in males but inhibitory in females (Ancel et al., 2017). The lack of effect of ovariectomy in females is similar to results obtained in mice (Iwasa et al., 2012; Quennell et al., 2010).

In conclusion, our data from this study indicate an increase in the level of expression of *KISS1* and a reduction in expression of *RFRP* around the time of puberty in ewes. *PDYN* expression is increased across the pubertal transition with no change in expression of *TAC3*. Because of the prominent role that kisspeptin is thought to play in puberty, we also measured *KISS1R* expression in GnRH cells prior to and after puberty, but there were no significant changes in the percentage of cells expressing the receptor and the expression of mRNA. In addition to the role that the genes investigated in this study play in the pubertal transition, other work strongly suggests that a fundamental switch in expression of transcriptional regulators are the drivers of puberty (Lomniczi et al., 2013). Further studies of such regulators would be warranted in a range of species if a comprehensive understanding of puberty is to be elucidated; these could include neuronal systems involved in brain sensing of bodyweight, which are also involved in regulation of reproduction. Expression of relevant receptors by Kisspeptin neurons and the level of input from modulatory afferents would also be informative. Finally, GDX had little effect on the expression of the genes of interest, suggesting that feedback effects of gonadal steroids at this time are different to those seen in the mature animal.

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## CONFLICT OF INTEREST

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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