Differential expression of CART in ewes with differing ovulation rates

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Abstract

We hypothesised that cocaine- and amphetamine-regulated transcript (*CARTPT*) would be differentially expressed in ewes with differing ovulation rates. Expression of mRNA for *CARTPT*, as well as *LHCGR*, *FSHR*, *CYP19A1* and *CYP17A1* was determined in antral follicles ≥ 1 mm in diameter collected during the follicular phase in ewes heterozygous for the Booroola and Inverdale genes (I+B+; average ovulation rate 4) and ++ contemporaries (++; average ovulation rate 1.8). In ++ ewes ($n=6$), *CARTPT* was expressed in small follicles (1 to <3 mm diameter), where $18.8 \pm 2.5\%$ follicles expressed *CARTPT*. CART peptide was also detected in follicular fluid of some follicles of ++ ewes. In I+B+ ewes, 5/6 ewes did not have any follicles that expressed *CARTPT*, and no CART peptide was detected in any follicle examined. Expression pattern of *CYP19A1* differed between I+B+ and ++ ewes with an increased percentage of small and medium follicles (3 to <4.5 mm diameter) but decreased percentage of large follicles (≥ 4.5 mm diameter) expressing *CYP19A1* in the I+B+ ewes. Many of the large follicles from the I+B+ ewes appeared non-functional and expression of *LHCGR*, *FSHR*, *CYP17A1* and *CYP19A1* was less than that observed in ++ ewes. Expression of *FSHR* and *CYP17A1* was not different between groups in small and medium follicles, but *LHCGR* expression was approximately double in I+B+ ewes compared to that in ++ ewes. Thus, ewes with high ovulation rates had a distinct pattern of expression of *CARTPT* mRNA and protein compared to ewes with normal ovulation rates, providing evidence for CART being important in the regulation of ovulation rate.

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Introduction

The number of follicles that develop for ovulation each reproductive cycle (i.e. ovulation rate) is a key determinant of fecundity. Ovulation rate varies considerably among mammalian species from those that predominately ovulate a single ova and thus have one offspring at a time such as humans and cattle to those that ovulate many ova at each reproductive cycle and thus have litters, such as pigs, mice and dogs. Although sheep are predominately a low ovulation rate species, ovulating one or two ova at each reproductive cycle, there is considerable plasticity in ovulation rate in sheep with some sheep ovulating over 10 ova at a reproductive cycle (Juengel *et al.* 2013). Factors such as nutrition, body size and genetics are known to influence ovulation rate in sheep (Scaramuzzi *et al.* 2011, Shorten *et al.* 2013).

Previous studies of the control of ovulation rate have focused on the role of gonadotropins from the pituitary gland (i.e. follicle-stimulating hormone (FSH) and luteinising hormone (LH)) because they are known to be critical for development of the ovarian follicles for

ovulation (Webb & Campbell 2007, Mihm & Evans 2008). The gonadotropins stimulate the growth of follicles and administering exogenous FSH can override the natural limitations on the number of ovulating follicles. This forms the basis for fertility treatments in livestock species (Barros *et al.* 2010, Menchaca *et al.* 2010) as well as humans (van Wely *et al.* 2011). Although ovulation rate has traditionally been thought to be controlled by the pituitary gland, increasing evidence points to local ovarian factors as key physiological regulators of ovulation rate (Gilchrist *et al.* 2004, Juengel & McNatty 2005, Silva *et al.* 2009). A search for novel ovarian factors that regulate fertility identified cocaine- and amphetamine-regulated transcript (*CARTPT*) as such a factor (Kobayashi *et al.* 2004, Yao *et al.* 2004). CART (the biologically active mature *CARTPT* peptide) was first identified as a neuropeptide with known roles in controlling body weight and reward response (Rogge *et al.* 2008). In contrast to the stimulatory role of the gonadotropins, CART appears to act as a suppressor of follicular growth, being actively expressed in follicles

undergoing atresia (i.e. dying) that will not go on to ovulate (Smith *et al.* 2011).

In cattle ovaries, *CARTPT* is expressed in both the oocyte and the supporting somatic cells (granulosa cells) of the follicle and regulates the production of oestradiol (Kobayashi *et al.* 2004, 2006), a key hormone involved in the timing of ovulation and receptivity of the female to the male (Fabre-Nys & Martin 1991, Christian & Moenter 2010, Flanagan-Cato 2011). Further, *CARTPT* is not expressed in the adult ovary in litter-bearing species, suggesting that this pathway is not active in animals with a high ovulation rate phenotype (Smith *et al.* 2011). Thus, the aim of the present study was to determine the expression pattern of *CARTPT* mRNA in antral follicles during selection of the ovulatory follicle and determine if the pattern of expression differs between sheep with a low or high ovulation rate phenotype. Furthermore, given the known interactions between *CART* and oestradiol synthesis and the responsiveness of follicles to gonadotropins, expression of mRNA encoding *LHCGR*, *FSHR*, *CYP17A1* and *CYP19A1* was also determined for these follicles.

Materials and methods

Animals

Ewes were sourced from the Invermay farm, and the experiment was approved by the Invermay Agricultural Centre's Animal Ethics Committee and followed the 1999 Animal Protection (Codes of Ethical Conduct) Regulations of New Zealand. All ewes were approximately 5.5 years old and were pasture-fed (grass) to meet the nutritional requirements and had free access to water. High ovulation rate ewes (I+B+), with a single copy of the Booroola mutation (*FecB*) and the Inverdale mutation (*FecX^l*) were identified through genotyping (GenomNZ, Mosgiel, New Zealand). On average, ovulation rate of I+B+ ewes is 258% that of ++ sheep (Juengel *et al.* 2013). Control (++) ewes were from a similar genetic background but did not carry either the Booroola or Inverdale mutations. The weight of the ewes was similar (64.5 ± 2.5 kg and 64.0 ± 1.7 kg for control and I+B+ ewes respectively). The study was performed during the natural breeding season.

Sample collection

Ewes ($n=6$ in each group) were presynchronised using controlled internal drug-release devices (CIDR-G; SVS Veterinary Supplies Ltd; Christchurch, New Zealand) administration for 13 days. After the removal of the CIDR, timing of onset of oestrus was determined by marking by a harnessed vasectomised ram with crayon marks evaluated approximately every 12 h. Prostaglandin $F_{2\alpha}$ analogue (PGF; 0.7 mL of Estrumate, active ingredient cloprostenol, SVS Veterinary Supplies Ltd) was given on days 5–6 of the subsequent oestrous cycle to induce the follicular phase and selection of preovulatory follicles. Ewes were killed by captive bolt and then exsanguinated 12 h after PGF injection.

Ovaries were collected, and all follicles ≥ 1 mm in diameter were dissected out of the ovaries, and follicular fluid was collected from follicles > 2 mm in diameter. After the addition of a protease inhibitor (Roche complete Mini Protease Inhibitor Cocktail Tablets; Sigma-Aldrich), follicular fluid was frozen on dry ice, whereas follicles were frozen in liquid nitrogen and both were stored at -70°C until further processing. A blood sample was collected by venipuncture from each ewe every 6 h for the 48 h preceding tissue collection. Plasma was collected from this sample and stored at -20°C until assayed.

Analysis of FSH and progesterone in plasma

Concentrations of FSH were measured in all plasma samples in a single RIA (McLeod *et al.* 1997) using reagents provided by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK; Bethesda, MD, USA). The sensitivity of the assay, defined as the lowest point on the standard curve, was 0.4 ng/mL and the coefficient of variation (CV) of 5 quality control pool samples with between 1 and 5 ng/mL FSH was $< 10\%$. Concentrations of progesterone were measured using a coat-a-count kit RIA (CAC kit, Siemens Healthcare Diagnostics) in the plasma sample collected at the time of administration of PGF and at tissue collection. The sensitivity of the assay, defined as the lowest point on the standard curve, was 0.08 ng/mL, and the CV of 3 quality control pool samples between 2 and 8 ng/mL was $< 5\%$. For the FSH and progesterone assay, no samples were below the sensitivity of the assay.

Measurement of mRNA encoding *CARTPT*, *LHCGR*, *FSHR*, *CYP19A1* and *CYP17A1*

The follicles were homogenised in TRIzol (Invitrogen, Life Technologies) using a Geno/Grinder 2010 (SPEX SamplePrep; Total Lab Systems Ltd, Auckland, New Zealand). Total cellular RNA was purified according to the manufacturer's protocol using the PureLink Pro 96 total RNA Purification Kit (Invitrogen, Life Technologies). Total cellular RNA (500 ng) was DNase I treated prior to cDNA synthesis using Superscript III (Life Technologies) according to the manufacturer's instructions. Three multiplexes were designed using Beacon Designer v8; *RPL19/CART*, *RPL19/FSHR/LHCGR* and *RPL19/CYP17A1/CYP19A1* (Table 1). Primers were purchased from Integrated DNA Technologies (Singapore), and the dual-labelled TaqMan probes were purchased from Sigma-Aldrich. Identity of amplified products was confirmed by sequencing (Waikato DNA Sequencing Facility, Hamilton, New Zealand). The sensitivity of the assay was optimised with a primer matrix approach for all genes using SYBR Green (Invitrogen, Life Technologies). The absence of non-specific products was assessed by dissociation curve analysis.

The concentration of the probe was optimised for all genes and assessed in the multiplex to confirm that the C_t values were not affected by multiplexing. For the *RPL19/CART* multiplex, there was a complete inhibition of *CARTPT* gene amplification; therefore, concentrations of *CARTPT* mRNA were measured using a SYBR Green method. Efficiency and range of each assay was calculated using serial dilutions of a standard sample (Table 2). Samples outside the range were

Table 1 Primer and probe sequences and concentrations used for the quantitative PCR assays.

Gene	Product size (bp)	Forward primer		Reverse primer		Probe		Dye
		Sequence	Final conc. (nM)	Sequence	Final conc. (nM)	Sequence	Final conc. (nM)	
<i>RPL19</i>	116	GACGATACCGTGAATCTA	200	CAGCTTGTGGATATGTTTC	300	TGTATCAGCCCTGTACCTGAAGG	150	Cy5
<i>FSHR</i>	112	CCTCCGATATCTGTTAATATC	300	GTGGATGTTTATATCTTGAATA	300	AACACAGGTATTAAGCACTTGCCAG	150	ROX
<i>LHCGR</i>	71	GAGTGATTCCTGGAAAA	400	GGATCAGTATTCAGACAAA	200	AAGCTAATGCTTTTGACAACTCC	150	HEX
<i>CYP17A1</i>	132	GATGGCAACCTGAAGTTA	300	GCTGATTATGTTGGTGAC	400	AAGATCATTATCAGGAAGCCAACTG	300	6FAM
<i>CYP19A1</i>	140	CTTGTTTGGAAATATGAGG	400	CTCCATACATCTTGTGTAG	400	CTCAATACCAAGGTCCCACTACTTT	300	HEX
<i>CART</i>	82	GAAGGAGCTGATTGAAGC	200	GCCATCTTCTCTCATAAATG	400			

Table 2 Characteristics of the quantitative PCR assays.

Gene	Efficiency (%)	R ²	Range (cycles)
<i>RPL19</i>	98.6	1.000	22.03–30.12
<i>CART</i>	99.0	0.988	25.35–33.50
<i>RPL19</i>	98.8	0.989	20.79–30.27
<i>FSHR</i>	99.7	0.995	23.71–32.95
<i>LHCGR</i>	98.8	0.990	23.08–31.91
<i>RPL19</i>	100.0	0.988	19.11–30.20
<i>CYP17A1</i>	99.7	0.992	21.64–32.70
<i>CYP19A1</i>	99.7	0.987	20.40–31.26

either repeated with a more dilute sample (those with Ct less than the lowest acceptable Ct) or were considered below the sensitivity of the assay (those with Ct greater than the highest acceptable Ct). All samples were run in duplicate with average Ct used for calculations. The average Ct for the reference gene, *RPL19*, did not differ between the two genotypes (i.e., I+B+ and ++ animals). A calibrator sample was run on every plate and was used to calculate the $\Delta\Delta Ct$ for each sample. Negative controls included no template reactions and samples that omitted the reverse transcriptase for cDNA synthesis. These controls gave the expected negative results for all assays.

Analysis of CART in follicular fluid

Concentrations of CART protein were measured in follicular fluid using a RIA (Phoenix Pharmaceuticals, Inc, Burlingame, CA, USA). This assay, which has 100% cross-reactivity with bovine CART (amino acids 42–89), has been validated for use with bovine follicular fluid (Kobayashi *et al.* 2004), and bovine and ovine CART are identical in this region. Very limited amounts of follicular fluid were available from the small follicles and thus the follicular fluid was diluted with assay buffer before being assayed. Sensitivity of the assay was 0.25–0.5 ng/mL (depending on the dilution of the follicular fluid used), and coefficients of variation for two standard samples at approximately 20 and 75% binding were <15%.

Statistical analysis

Statistical analysis was undertaken using GENSTAT, version 15 (VSN International, Hemel Hempstead, UK). Total ovarian weight, the number of CL, average weight of CL and total CL weight were examined using analysis of variance. The numbers of follicles classified as small (1 to <3.0 mm in diameter), medium (3 to <4.5 mm in diameter) and large (≥ 4.5 mm in diameter) as well as total number of follicles ≥ 1 mm in diameter were transformed (ln for large and total, ln(number+1) for small and medium as some animals had no follicles of this size) prior to examination by analysis of variance. Concentrations of FSH and progesterone in plasma were analysed using REML taking into account the repeated nature of the samples. An autoregressive structure (AR(1)) was used for FSH concentrations with a uniform structure used for progesterone analysis. For analysis of mRNAs of interest, follicles were classified into one of the three categories: small, medium or large. These size classes were chosen to represent follicles that were gonadotropin responsive but not yet gonadotropin dependent (small), gonadotropin dependent

(medium) and forming the presumptive preovulatory pool (large). Proportion of follicles at each size class expressing the mRNA was compared between genotypes using GLIM with binomial distribution and a logit link. Fold-difference of mRNA concentrations was examined using REML including genotype, follicle size and genotype by follicle size interactions as fixed effects with animal as a random factor. For *CYP17A1*, *LHCGR* and *FSHR* mRNA, values below the detection limit of the assay were included as 0. Data were transformed (natural log(fold-difference+1), to allow for inclusions of 0 values) prior to analysis for normalisation. For *CYP19A1* mRNA, analysis of fold-differences was limited to those follicles expressing *CYP19A1* mRNA given that a high proportion of follicles did not have detectable *CYP19A1* mRNA. Data were transformed (natural log(fold-difference)) prior to analysis for normalisation. For all mRNAs, a significant follicle size by genotype interaction was observed ($P<0.001$), and thus, difference between genotypes were analysed for each follicle size. Differences in expression of *CYP17A1*, *LHCGR* and *FSHR* mRNA between *CARTPT*-positive and -negative follicles of the ++ animals were analysed using REML with animal as a random factor. Data presented are the geometric means and 95% confidence limits of the back transformed data unless otherwise stated.

Results

Ovarian and endocrine characteristics

Total ovarian weight was not different between genotypes ($P=0.129$; Table 3). The total number of follicles were decreased ($P=0.004$) in the I+B+ ewes compared to the ++ ewes. This was the result of a decreased ($P=0.004$) number of small follicles, with no differences observed in the number medium or large follicles (Table 3). When compared to ++ ewes, the I+B+ ewes had an increased ($P=0.008$) number of CL with each CL weighing less ($P<0.001$), resulting in no observed differences ($P=0.144$) in total CL weight (Table 3).

Concentrations of FSH were increased ($P<0.001$) in the I+B+ compared to the ++ animals (Table 3).

Sampling time did not affect the concentrations of FSH in plasma ($P=0.113$); thus, there was no evidence of a sustained decrease in FSH concentrations, as would be expected if dominant follicles were present on the ovary prior to the initiation of the follicular phase. Concentrations of progesterone in plasma were similar between genotypes both before and 12 h after the initiation of the follicular phase (Table 3). As expected, concentrations of progesterone were decreased 12 h after prostaglandin administration ($P<0.001$), with progesterone concentrations decreasing by at least 60% in all animals, indicating that administration of PGF had induced luteal regression in all animals.

Pattern of expression of mRNA encoding *CARTPT*, *LHCGR*, *FSHR*, *CYP19A* and *CYP17A* in follicles

CARTPT

The majority of follicles did not contain detectable amounts of *CARTPT* mRNA. The *CARTPT* mRNA was predominately expressed by small follicles in the ++ animals, where all animals had some follicles expressing *CARTPT* mRNA with an average of $18.8\pm 2.5\%$ (range 13–22%) of follicles positive for *CARTPT* in this size class (Fig. 1). In the I+B+ animals, from the 113 follicles in which RNA extraction was successful, expression of *CARTPT* was limited to two large follicles collected from a single animal (Fig. 1). No small follicles expressed *CARTPT* from this genotype. In no instance was a follicle that was positive for *CARTPT* also positive for *CYP19A1* mRNA expression.

LHCGR and *FSHR*

Most follicles in all size classes expressed mRNA for both the *LHCGR* and the *FSHR* with no differences in proportion of follicles expressing either mRNA between genotypes for small and medium follicles. There was a decreased ($P<0.05$) percentage of large follicles in the I+B+ animals expressing *FSHR* and *LHCGR* mRNA, whereas all large follicles expressed both mRNAs in the

Table 3 Ovarian and endocrinological characteristics of ewes.

Characteristic	Genotype		Pooled S.E.M.	P value (++ vs I+B+)
	++	I+B+		
Total ovarian weight (g)	3.73	2.92	0.35	0.129
Ln(total number of follicles ≥ 1 mm)	3.83	2.86	0.26	0.004
Ln(number of small follicles (1 to <3 mm))	3.69	2.40	0.34	0.004
Ln((number of medium follicles (3 to <4.5 mm)) + 1)	1.35	1.42	0.40	0.854
Ln((number of large follicles (≥ 4.5 mm)) + 1)	1.22	0.88	0.35	0.349
Number of CL	1.8	4.0	0.5	0.008
Ave weight of CL (g)	0.53	0.17	0.05	<0.001
Total CL weight (g)	0.94	0.63	0.14	0.144
Average FSH (ng/mL)	1.11	2.98	0.30	<0.001
P4 at PGF (ng/mL)	2.87	2.75		
P4 at tissue collection (ng/mL)	0.82	0.82	0.34	0.49

P4, progesterone.

Figure 1 Size distribution of follicles negative (Neg) or positive (Pos) for *CARTPT* mRNA expression in ++ and I+B+ ewes.

++ animals, an on average $77 \pm 7\%$ expressed *FSHR* and $92 \pm 3\%$ expressed *LHCGR* in the I+B+ animals.

When examining the level of expression, no differences were observed between genotypes for small and medium follicles for *FSHR* mRNA expression (Fig. 2). Expression of *LHCGR* mRNA was increased approximately 2-fold in small and medium follicles of I+B+ animals compared to ++ animals. In contrast, large follicles of I+B+ animals had decreased concentrations of both *FSHR* and *LHCGR* compared to large follicles from ++ animals (Fig. 2).

CYP17A1 and *CYP19A1*

Most small and medium follicles were positive for expression of *CYP17A1* with no differences observed in the average proportion of follicles expressing *CYP17A1* between genotypes in these classes. In ++ animals, all large follicles expressed *CYP17A1*, whereas on average, only $54 \pm 9\%$ of large follicles expressed *CYP17A1* mRNA in the I+B+ animals ($P < 0.001$).

In ++ animals, *CYP19A1* mRNA was expressed in a few small follicles, with increased expression in medium follicles, and the majority of large follicles expressed *CYP19A1* mRNA (Fig. 3). The pattern of expression was very different in the I+B+ animals, with a greater percentage of small and medium follicles expressing *CYP19A1* mRNA than ++ animals. The majority of the medium follicles expressed *CYP19A1* mRNA, but relatively fewer of the large follicles expressed *CYP19A1* when compared to ++ follicles (Fig. 3).

Of those follicles expressing *CYP19A1* mRNA, expression was increased in small follicles and tended to be increased in medium follicles collected from I+B+ animals compared to those from ++ animals (Fig. 2). Expression of *CYP17A1* mRNA also tended to be increased in small follicles of I+B+ ewes (Fig. 2). For large follicles, expression of both *CYP17A1* and *CYP19A1* mRNA was decreased in I+B+ compared to ++ animals (Fig. 2).

Figure 2 Expression of mRNA encoding *LHCGR*, *FSHR*, *CYP17A1* and *CYP19A1* in small (1 to <3 mm in diameter, top panel), medium (3 to <4.5 mm in diameter, middle panel) and large (≥ 4.5 mm in diameter, bottom panel) ovarian follicles from ++ and I+B+ ewes. Values are geometric means and 95% confidence intervals. I+B+ different to ++ indicated by $**P < 0.01$, $***P < 0.001$ or $^aP < 0.1$.

Characterisations of *FSHR*, *LHCGR* and *CYP17A1* mRNA in *CARTPT*-positive and -negative follicles

To further characterise the potential interactions between *CARTPT* expression and gonadotropin responsiveness and steroid production, relative expression of *FSHR*, *LHCGR* and *CYP17A1* mRNAs were compared between small follicles that were positive or negative for *CARTPT* mRNA in the ++ control ewes. Concentrations of *FSHR* mRNA were increased in *CARTPT*-positive follicles compared to those in *CARTPT* negative follicles, with no differences observed in *LHCGR* or *CYP17A1* mRNA (Fig. 4).

Figure 3 Percentage (mean $\pm$ S.E.M.) of follicles in each size class expressing CYP19A1 mRNA in small (1 to <3 mm in diameter), medium (3 to <4.5 mm in diameter) and large ($\geq$ 4.5 mm in diameter) follicles from ++ and I+B+ ewes. *Expression in I+B+ follicles is different ($P < 0.01$) than expression in ++ follicles.

Concentrations of CART peptide in follicular fluid

Only 11 of 113 follicles examined had detectable amounts of CART peptide in their follicular fluid. Detection of CART peptide in the follicular fluid was limited by the amount of follicular fluid available for analysis in the small follicles as only 4/12 follicles with detectable expression of *CARTPT* mRNA had detectable CART peptide in their follicular fluid. The follicular fluid containing CART peptide was from follicles from the ++ ewes and ranged in size from 2.2 to 3.1 mm in diameter. These included follicles that were positive for *CARTPT* mRNA as well as some that were negative. No follicles (0/43) from the I+B+ ewes had detectable CART peptide in their follicular fluid. No follicle (0/48) that

Figure 4 Relative expression of mRNA (geometric mean and 95% confidence intervals) encoding *LHCGR*, *FSHR* and *CYP17A1* in *CARTPT*-positive (Pos) or *CARTPT*-negative (Neg) small follicles (1 to <3 mm in diameter) from ++ sheep. Expression in *CARTPT*-negative follicles different than *CARTPT*-positive follicles are indicated by *** $P < 0.001$ or ^a $P < 0.1$.

expressed *CYP19A1* mRNA had detectable amounts of CART peptide.

Discussion

In the ++ sheep, *CARTPT* mRNA expression was only observed in follicles <3 mm in diameter and only in approximately 20% of the follicles in that class range. One previous study examining *CARTPT* expression in sheep follicles collected from the slaughterhouse observed some expression in follicles greater than 3 mm in diameter (smallest size examined), with the level of expression decreasing as follicle size increased (Huang *et al.* 2016). The differences between these studies are unclear but could be related to the sensitivity of the assays, breed of sheep (genetics), timing of follicular collection or nutritional status of the sheep. However, taking the results together, it appears that *CARTPT* mRNA decreases as the follicle matures such that the highest expression is in follicles less than 3 mm in diameter, which are gonadotropin responsive but not dependent.

In cattle and sheep, treatment of granulosa cell cultures with CART caused decreased production of oestradiol (Kobayashi *et al.* 2006, Huang *et al.* 2016), which in cattle has been linked to a decrease in expression of *CYP19A1* mRNA (Sen *et al.* 2007). Furthermore, injection of CART into the follicular fluid of dominant follicles reduces oestradiol secretion and *CYP19A1* mRNA concentrations, leading to the hypothesis that CART is an important regulator of oestradiol secretion in cattle (Lv *et al.* 2009). The timing of expression of *CARTPT* during the development of sheep follicles does not support the regulation of oestradiol synthesis as a major direct function of CART in sheep as no follicles were observed that expressed both *CYP19A1* mRNA and *CARTPT* mRNA/CART peptide. However, it is possible that expression of *CARTPT*/CART peptide prevents the follicles from being able to express *CYP19A1*. Given the known role of oestrogens in stimulating granulosa cell proliferation and preventing atresia (Quirk *et al.* 2004), expression of *CARTPT* could target a follicle for an atretic pathway. Furthermore, lack of expression of *CARTPT* in the I+B+ follicles could underlie a reduced level of atresia in these animals thus allowing more ovulatory follicles to develop and increasing ovulation rate. However, it is unclear if atresia rates are suppressed in ewes with the Booroola or Inverdale genes, with some studies failing to find differences in the overall rates of atresia (McNatty *et al.* 1985, Shackell *et al.* 1993) and others finding differences in the pattern of atresia and level of atresia in specific follicular size classes (Driancourt *et al.* 1985, Mandiki *et al.* 2000). However, it should be noted that the timing of the majority of follicle loss by atresia is typically earlier in the development of ovarian follicles in sheep, with greater

than 85% of follicles classified as early atretic being <1.6 mm in diameter in Merino ewes (Driancourt *et al.* 1985). Thus, if CART is affecting atresia, it may be targeting follicles later in the development, potentially influencing the pool of follicles available for selection for ovulation. In support of this, the rate of atresia in large (>5.0 mm where average size of B+ ovulatory follicles was 5.7 mm), but not small or medium, follicles of ewes carrying a single copy of the Booroola gene was reduced compared to ++ controls (Mandiki *et al.* 2000). Although *CARTPT* expression occurs prior to this, it is possible that the expression of *CARTPT* prevents the expression of aromatase and production of oestradiol, leading to atresia at a later stage of development.

Surprisingly, expression of *FSHR* mRNA was increased in small follicles expressing *CARTPT* mRNA compared to those not expressing *CARTPT*. This observation is inconsistent with previous observations in bovine granulosa cells where FSH suppresses *CARTPT* mRNA expression and CART suppresses FSH signalling (Sen *et al.* 2007, 2008, Lv *et al.* 2009). Similarly, in ovine granulosa cells, FSH stimulated cell proliferation in long-term cultures (7 days), but inclusion of CART in the media blocked this stimulation (Huang *et al.* 2016). Additionally, in cattle, expression of *CARTPT* mRNA is increased in atretic antral follicles when compared to that in healthy antral follicles (Smith *et al.* 2011). However, in sheep, even very atretic follicles continue to express *FSHR* mRNA (Tisdall *et al.* 1995), and thus, potentially, the ability of CART to suppress FSH actions in ovine granulosa cells occurs at a point distal to *FSHR* mRNA expression.

Interestingly, most animals with high ovulation rate had no detectable expression of *CARTPT* mRNA in follicles ≥ 1 mm, with no animal having any follicles positive for CART protein. This contrasts to what was observed in the ++ ewes, where *CARTPT* mRNA was observed in follicles 1–3 mm in diameter for all animals and most animals had follicles with detectable CART peptide in the follicular fluid of at least one follicle of this size class. Thus, altered expression of *CARTPT* mRNA was associated with increased ovulation rates.

It is also important to note that follicles mature at a smaller size in both Booroola and Inverdale ewes. For instance, the average size of the preovulatory follicle is smaller (McNatty *et al.* 1985, Shackell *et al.* 1993) and oocytes exit their more rapid growth phase earlier, at a follicular diameter of 0.24 mm in BB ewes compared to that of 0.38 mm in ++ ewes (Wilson *et al.* 2001). Thus, follicles of similar size may not represent follicles of similar maturity when comparing ++ to I+B+ ewes (Juengel *et al.* 2013), and this could underlie the observed differences in expression in *CARTPT* mRNA and CART peptide, with the potential that follicles <1 mm in diameter express *CARTPT* mRNA in I+B+ ewes. However, the expression of *CARTPT* mRNA and CART peptide was not observed in the ++ ewes until

follicles reached a diameter of at least 1.5 mm and continued to be observed in follicles until they were 3 mm in diameter. Thus, it is likely that some of the I+B+ follicles examined would be of similar maturity of the follicles expressing *CARTPT*/CART peptide in the ++ ewes.

Whether altering activities of either BMP15 or the BMP signalling pathway might directly alter *CARTPT* expression in the I+B+ ewes is unknown. *BMP15* is known to be produced from the oocyte of type 2 (primary) and larger follicles with expression increasing during preantral follicle development (Feary *et al.* 2007). Additionally, granulosa and theca cells both produce mRNA encoding *BMP1B* and *BMP2* (Feary *et al.* 2007), the proposed receptors for BMP15 (Juengel *et al.* 2013). In cattle, addition of CART to the media was able to suppress BMP2-stimulated oestradiol production (Selvaraju *et al.* 2013), indicating a potential interaction between these pathways.

CART has known roles in regulating the body weight (Rogge *et al.* 2008), and in the mouse pituitary, restricted feeding leads to an upregulation of *Cartpt* mRNA (Burgos *et al.* 2016) providing evidence that in some tissues, poor nutrition would result in increases of *Cartpt* mRNA. In sheep, it is well known that providing an increasing level of nutrition just before breeding results in an increased number of lambs born through increased ovulation rates (Scaramuzzi *et al.* 2006). Potentially, nutritional suppression of *CARTPT* in granulosa cells could be a pathway for nutritional increases in ovulation rate in sheep, but this hypothesis remains to be tested. Interestingly, we have previously shown that ewes heterozygous for the Inverdale mutation (i.e. I+) have an increased sensitivity to nutritional regulation of ovulation rate (Dammers *et al.* 2011). Similar increased sensitivity to nutritional regulation of ovulation rate has been observed in Booroola ewes (Landau *et al.* 1995), although this effect has not always been apparent (Montgomery *et al.* 1983). Further investigation into the potential role of CART in regulating the nutritional control of ovulation rate in sheep seems warranted.

Concentrations of FSH were also higher in the I+B+ animals. Although previous studies in Inverdale and Booroola animals have reported variable results regarding FSH concentrations with some studies showing no differences between ++ and Booroola ewes (Driancourt *et al.* 1991, Souza *et al.* 1997), previous studies with New Zealand-sourced Booroola sheep have shown consistently increased FSH concentrations (McNatty *et al.* 1987, 1989, 1994, Phillips *et al.* 1993). Given that FSH is known to suppress *CARTPT* mRNA, increased FSH concentrations could be suppressing the expression of *CARTPT* to non-detectable levels in these animals, thus altering the number of follicles available for the selection for ovulation.

Previous studies have shown that ewes carrying either the Inverdale or Booroola gene have follicles

that mature at a smaller size (Juengel *et al.* 2013). This is linked to earlier responsiveness to LH, but not FSH, in the granulosa cells of these follicles (McNatty *et al.* 2009, Crawford *et al.* 2011). The increased expression of mRNA for *LHCGR*, but not *FSHR*, in small and medium follicles is consistent with these findings. Additionally, the differing pattern of *CYP19A1* mRNA expression is also consistent with follicles maturing at a smaller size. The observation of large follicles in the I+B+ animals was somewhat unexpected as ewes homozygous for the Booroola mutation typically have very few, if any, follicles in this size class. However, most of these follicles expressed little or no mRNA encoding *FSHR*, *CYP17A1* or *CYP19A1*, indicating that they were likely cystic and not functional.

In conclusion, in ++ ewes, *CARTPT* mRNA was expressed in approximately 20% of ovine follicles from 1 to 3 mm in diameter, and CART peptide was also observed in the follicular fluid of some of the follicles. No follicles expressed both *CARTPT* (mRNA or peptide) and *CYP19A1* mRNA; therefore, the role of CART in sheep may differ to that observed in cattle, where a key role of CART appears to be the suppression of oestradiol synthesis in subordinate follicles during the selection of the dominant follicle. Most animals with high ovulation rates had non-detectable levels of *CARTPT* mRNA in any follicle ≥ 1 mm in diameter, and CART peptide was not observed in the fluid of any follicle examined in these animals. Thus, *CARTPT* mRNA/CART peptide expression may be an important regulator of the number of follicles selected for ovulation in sheep.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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