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Gene expression of GnRH, kisspeptin, neuropeptide Y and receptors for estrogen and leptin in the hypothalamus of suckled and weaned beef cows

(Ekspresi gen GnRH, kisspeptin, neuropeptida Y dan reseptor untuk estrogen dan leptin di dalam hipotalamus lembu pedaging yang menyusu dan dicerai susu)

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Abstract

The aim of this study was to ascertain the expression of multiple genes in the hypothalamic sub-regions of postpartum beef cows that were suckled or weaned, and had different ovarian phenotypes. Genes studied were gonadotrophin-releasing hormone (*GNRH1*), kisspeptin (*KISS1*), estrogen receptor-α (*ESR1*), leptin receptor (*LEPR*) and neuropeptide Y (*NPY*) in two hypothalamic sub-regions, H1 (anterior) and H2 (ventral posterior). The expression of *GNRH1* was around 7-fold greater ($p < 0.01$) within H1 than H2 while the expression of KISS1 was around 20-fold greater ($p < 0.01$) within H2 than H1. Both *ESR1* and *NPY* showed greater ($p < 0.05$) expression within H2 than H1. Weaned cows had lower ($p < 0.05$) expression of *ESR1, LEPR* and *NPY* than suckled cows in H1. Cows with suppressed follicle growth (follicles 4 – 5 mm) had greater ($p < 0.05$) *LEPR* expression within H1 compared to cows with active follicle growth (follicles 10 mm) and cows that had ovulated. This is the first report of *KISS1* expression in the bovine brain and also of regional differences in the expression of multiple genes within the bovine hypothalamus. The study on simultaneous gene expression within the hypothalamus provides a new approach in understanding the complex gene networks linked to the effects of nutrition, lactation, ovarian steroids, metabolic homeostasis and maternal bonding on central mechanisms associated with postpartum reproduction in bovine.

Introduction

The postpartum anoestrus period in lactating beef cows results from the negative influences on reproductive function imposed by the nutrient requirements of lactation (Lucy 2003; Roberts et al. 2005), suckling stimulus (Wettemann et al. 2003), maternal bond (Silveira et al. 1993; Montiel and Ahuja 2005), ovarian steroids (Savio et al. 1990) and negative energy balance (Montiel and Ahuja 2005; Roberts et al. 2005; Shrestha et al. 2005). It has been proposed

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that the various factors which contribute to postpartum anoestrus in cows are integrated centrally at the hypothalamus through common pathways that regulate the secretion of gonadotrophin-releasing hormone (GnRH) (Short et al. 1990). According to this concept, the resumption of ovulation after calving is dependent on the return of GnRH secretion, and hence the follicle secreting hormone (FSH) and luteinising hormone (LH) secretion, and the patterns required for full maturation and ovulation of the dominant ovarian follicle (Daftary and Gore 2004).

An alternate model for postpartum reproduction in cows proposes that the return to ovulation after calving is determined at the level of the ovaries by mechanisms that involve intra-ovarian molecules, inter-ovarian molecules and sensitivity to LH (Diskin et al. 2003). In the latter model, an increase in sensitivity of the dominant follicle to LH, independent of changes in LH secretion, results in an increase in estrogen-17β (estrogen) production by the dominant follicle to amounts required to induce the pre-ovulatory surge release of LH. The two models for resumption of ovulation in the postpartum cow need not be mutually exclusive and both central (hypothalamic) and somatic (ovarian) tissue mediated mechanisms could be involved.

The relative contribution of central and somatic mechanisms to the resumption of ovulation postpartum may differ for beef and dairy cattle due to marked differences in lactation demand and most likely differ considerably in general metabolic homeostasis (Lucy 2003). Alternatively, the primary mechanism could be related to the prevailing metabolic status based on lactation demand and other factors. The resumption of postpartum ovulation in beef cows can be induced by weaning, provided that the cows are in an appropriate body condition (Wettemann et al. 2003). The mechanisms whereby a calf suppresses reproduction could include, singularly or in combination, the nutrient requirements of milk production, suckling stimulus, maternal bond and metabolic homeostasis.

Evidence at the hypothalamic level for interrelationships between metabolic homeostasis and reproduction includes the presence of leptin receptors on kisspeptin and neuropeptide Y (*NPY*) neurons (Messager et al. 2005; Gamba and Pralong 2006). Based on this information, the present study was undertaken to determine the expression of *GNRH*, kisspeptin and *NPY* genes and receptor genes for estrogen and leptin, in the hypothalamus of suckled and weaned beef cows. Weaning was used to induce increased ovarian follicular growth and ovulation, and hypothalamic gene expression was compared for cows of different ovarian phenotypes. This study was the first to report multiple gene expression within the bovine hypothalamus.

Materials and methods

Animals and experimental design

The study was approved by The University of Queensland Animal Ethics Committee (SAS/719/06 /CRC). Cows used in the study were kept on pasture and received standard management except when required for experimentation.

Three-year-old first-parity Zebu cows (*Bos indicus*, Brahman breed) were chosen for the study as this breed typically has an extended period of anoestrus when lactating. The cows were managed so that they were in relatively good body condition as reflected by body condition score (BCS) between 3.5 and 4.0 on a scale of 1.0 to 5.0. Assessment of BCS is based on the animal's weight for age and weight for height ratios, as well as its relative proportions of muscle and fat. The amount of tissue cover between the points of the hip, over the transverse processes of the lumbar vertebrae, the cover over the ribs and the pin bones below the tail are taken into consideration.

Cows were maintained in this body condition so that, to the extent possible, the presence of a calf (i.e. nutrient demand

of lactation, suckling stimulus, maternal bonding) was the major (and possibly only) constraint to the resumption of ovulation postpartum. Six cows were weaned between day 20 and 37 of postpartum and slaughtered either 6 (n = 2) or 13 (n = 4) days after weaning. On each day of slaughter, complementary cows that continued to suckle a calf, and matched by days of postpartum, were also slaughtered (n = 6). The latter cows were separated from their calves around 18 to 24 h before slaughter.

Brain collection and sectioning

Cows were slaughtered at a commercial abattoir using the non-penetrating captive bolt technique and brains were recovered within 45 to 90 min of slaughter. Brains were initially sectioned using a medial sagittal incision to reveal the hypothalamus in each hemisphere. The hypothalamic region on each side of the brain was dissected into 3 sub-regions classified as H1, H2 and H3 as shown in *Figure 1*. To ensure standardised sampling, the optic chiasma (OC), mammillary body (MB), thalamus (T) and the pituitary stalk were used as references. Each sub-section was cut to a depth of 3 – 4 mm.

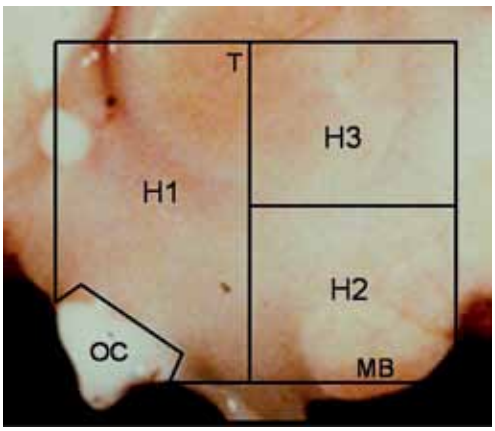

Figure 1. Hypothalamic sub-regions (H1, H2, H3) used to determine regional gene expression in the bovine brain. Note: OC = Optic chiasma; MB = Mammillary body; T = Thalamus

Regions and nuclei included in sub-region H1 were the suprachiasmatic-preoptic area, anteroventral periventricular nucleus, anterior hypothalamic nucleus, anterior portion of the arcuate nucleus, nearby areas of the diagonal band of Broca and medial septum. Sub-region H2 included the medial basal hypothalamus-median eminence, ventromedial hypothalamus, posterior portion of the arcuate nucleus and anterior part of the mammillary body. Sub-region H3 included the posterior hypothalamus, dorsomedial hypothalamus, lateral hypothalamus and ventral portion of the thalamus. Brain tissue was placed in RNAlater (Qiagen, Victoria, Australia) on ice for transport to the laboratory and kept at 4 °C overnight before storage at –20 °C or –80 °C until required for ribonucleic acid (RNA) extraction.

Ovarian phenotypes

The cows were divided into three groups based on their ovarian phenotype at slaughter. Cows in Group 1 (ovarian phenotype 1, OP1) had suppressed ovarian follicular growth with follicles of maximum diameter of 4 – 5 mm, which in cattle is the size at which follicles are gonadotrophin dependent. Cows in Group 2 (OP2) had increased follicular growth to a diameter of around 10 mm but without ovulation, and cows in Group 3 (OP3) had a corpus haemorrhagicum and/or corpus luteum at slaughter. Ovarian phenotype 1 (n = 4) comprised only suckled cows; OP2 (n = 4) comprised two suckled cows and two 13-day weaned cows; and OP3 (n = 4) comprised only 13-day weaned cows.

RNA extraction and reverse transcription

Total RNA was extracted using the RNeasy extraction kit (Qiagen, Victoria, Australia) with the following modifications: (i) approximately 400 mg of hypothalamic tissue was homogenized in lysis buffer (RLT) and centrifuged at 3000 x g for 10 min and (ii) 1/10 volume of chloroform:isoamyl alcohol (24:1) was

added to the supernatant and centrifuged 12,000 x g at 4 °C for 10 min. Extracted RNA was stored at –80 °C until required. RNA integrity number (RIN) was verified using the Bioanalyzer (Agilent Technologies, Victoria, Australia). Residual genomic DNA contamination was removed with the DNA free kit (Ambion, Texas, USA).

A mixture of 500 ng Oligo (dT) $_{12–18}$ (Invitrogen, California, USA), 300 ng random primer oligonucleotides (Invitrogen) and 200U Superscript III Reverse Transcriptase (Invitrogen) were added to 1 μg of DNA-free RNA and reverse transcribed in storage buffer at 50 °C for 1 h. The cDNA was then subjected to a final purification using the QIAquick PCR Purification kit (Qiagen). The consistency of cDNA quality was verified in ethidium-bromide stained 1% agarose gel electrophoresis.

Quantitative real time polymerase chain reaction (qRT-PCR)

Specific primers were designed to *Bos taurus* sequences using the Primer3 software (Rozen and Skaletsky 2000). The nucleotide sequences of each primer pair are shown in *Table 1*. All reactions were performed with 2X SYBR Green I Master Mix Buffer (Applied Biosystems, CA, USA) and 400 nM of primers. Approximately 10 ng of cDNA was used for each reaction which was run on the ABI PRISM 7900HT Sequence Detector (Applied Biosystems) and each sample was assayed in triplicate. Replicates were dispensed with the Biomek 2000 Lab Automation Workstation (Beckman Coulter, New South Wales, Australia).

The following cycling conditions were applied for amplification: Step 1 = 50 °C for 2 min; Step 2 = 95 °C for 10 min; Step 3 = 95 °C for 15 s and 60 °C for 1 min. Step 3 was repeated for 40 cycles. A melt curve analysis step was included (95 °C for 15 s, 60 °C for 15 s and 95 °C for 15 s) to confirm the specificity of the reaction. Furthermore, PCR products were separated on agarose gel

Table 1. Primers used for real time qRT-PCR

Gene		Forward primer (5'–3')	Reverse Primer (5'–3')
GNRH1	Gonadotrophin releasing hormone 1	GGGGACTCTCTGCTCTCAAA	TGCCCAGTTTCCTCTTCAAT
KISS1	Kisspeptin	ATGAACGTGCTGCTTTCCTG	CGAGCCTGTGGTTCTAGGATT
ESR1	Estrogen receptor α	ATGATGAAAGGCGGAATACG	AAGGTTGGCAGCTCTCATGT
NPY	Neuropeptide Y	CAGGCAGAGATACGGGAAAC	GGGAGGACTGGCAGACTCTA
LEPR	Leptin receptor (long signaling form)	GGCCTGGATGAACTTTTGAA	TGTGAGCAACTGTCCTGGAG
YWHAZ	Tyrosine3-monooxygenase /tryptophan monooxygenase activation protein, zeta polypeptide	ACCTACTCCGGACACAGAACAT	GAAGATTCCTCTCCTCATTGGA

and visually inspected, and the identity of the amplicon was verified by sequencing.

Normalisation of PCR data

All genes were normalised against tyrosine3-monooxygenase/tryptophan monooxygenase activation protein, zeta polypeptide (*YWHAZ*) which was stably expressed across all animals and hypothalamic tissues by the web-based software GeNorm (Vandesompele et al. 2002). PCR efficiencies (E) for each gene were calculated using the LinReg PCR analysis program (Ramakers et al. 2003). The average of the triplicate readings was normalised to obtain relative expression of each gene (Pfaffl 2001).

Statistical analyses

All statistical analyses were carried out using $\log_{10}$ transformed data to achieve normal distribution with the R software (Team 2006). In all gene expression experiments, analyses for treatment and ovarian phenotype effects were tested separately. In addition, hypothalamic sub-regions, treatment, ovarian phenotype, day of collection and days postpartum, were fitted as fixed variables in a linear model before factorial analysis of variance (ANOVA). Significance was set at $p < 0.05$. The differences between hypothalamic sub-regions were determined using the Bonferroni Multiple Test. The differences between means for the factorial levels i.e. ovarian phenotype and treatment were tested by one-way ANOVA.

Correlation tests were carried out using Spearman's product moment correlation. Before the correlation tests, data distribution were visualised to identify any confounding effects. The following correlations were tested: (i) correlation between the relative expression values of two candidate genes for 12 cows separately for sub-region H1 and H2 and (ii) correlation between the relative gene expression values and RNA integrity number (RIN) values for all genes mentioned above. Spearman's rank correlation coefficient (ρ) was established. An H2 sample of a weaned cow in OP2 and an H3 sample of a suckled cow in OP1 were excluded from the analysis due to technical variability.

Results and discussion

Effect of sampling on RNA integrity

RNA integrity number (RIN) values ranged from 6.4 – 7.5 and there was no correlation between RIN value and relative gene expression (data not shown). The RIN information indicated that the hypothalamic sampling and processing procedures did not compromise the integrity of mRNA.

Regional gene expression

The normalised expression of all genes was low within hypothalamic sub-region H3 (*Table 2*). Given the low gene expression within sub-region H3, this region was excluded from further analyses and consideration. *GNRH1, KISS1, ESR1* and *NPY* showed differential expression between sub-regions H1 and H2. The expression of *GNRH1* within H1 was around 7-fold greater ($p < 0.01$) than expression within H2 whilst *KISS1* expression within H2 was around 20-fold greater ($p < 0.01$) than the expression within H1 (*Table 2*). *ESR1* and *NPY* both had greater ($p < 0.05$) expression within H2 than H1, although absolute expression for the two genes differed (*Table 2*).

Effect of weaning on ovarian activity

Cows that continued to suckle a calf had ovaries with suppressed ovarian follicular growth (follicles 4 – 5 mm) except for two suckled cows that showed evidence of increased follicular growth (follicles 10 mm). The latter two cows were at postpartum day 51 and day 57 at the time of slaughter. The ovaries of two cows weaned 6 days before slaughter showed increased follicular growth (follicle sizes up to 10 mm). These cows were at day 26 and day 28 postpartum at the time of slaughter. The ovaries of four cows weaned 13 days before slaughter had either a corpus haemorragicum or corpus luteum. These cows were at days

33, 35, 46 and 50 postpartum at the time of slaughter. Hence, cows weaned between day 20 and 37 postpartum had increased ovarian follicular growth within several days of weaning and ovulation occurred between 7 and 13 days after weaning.

Weaning and gene expression

Weaning was associated with lower (p <0.05) expression of *ESR1, LEPR* and *NPY* within sub-region H1, and there was no significant effect (p >0.05) of weaning on the respective expression of these genes within sub-region H2 (*Table 3*). There was also no significant effect (p >0.05) of weaning on the expression of *GNRH1* and *KISS1* within either sub-region H1 or H2. A feature of gene expression was the relatively large variation between samples within weaned and suckled cows (e.g. *GNRH1* for sub-region H1 and *KISS1* for sub-region H2).

Table 2. Gene expression levels (mean ± SEM) in hypothalamic sub-regions H1, H2 and H3 for primiparous Brahman cows

Gene	Hypothalamic sub-region		
	H1 (n = 12)	H2 (n = 11)	H3 (n = 11)
GNRH1	350 ± 105a	50.6 ± 32.6c	0.9 ± 0.2b
KISS1	44.4 ± 14.8a	976 ± 335c	1.5 ± 0.5b
ESR1	8.1 ± 1.2a	19.3 ± 4.2c	1.3 ± 0.3b
NPY	13.5 ± 2.1a	25.4 ± 4.0c	1.6 ± 0.4b
LEPR	67.0 ± 9.8a	159 ± 35a	9.4 ± 5.1b

Mean values with similar letters within rows are not significantly different (p <0.05)

Ovarian phenotype and gene expression

The expression of *LEPR* within sub-region H1 was greater (p <0.05) for cows with ovarian phenotype OP1 (99.3 ± 10.6) than in cows with ovarian phenotype OP2 (59.2 ± 10.7) and OP3 (42.6 ± 16.4) (*Table 4*). There was a similar trend for LEPR expression within sub-region H2 but this was not significant. There were no significant differences in the expression of *GNRH1, KISS1, ESR1* and *NPY* between ovarian phenotypes within sub-regions H1 and H2. As noted above, a feature of gene expression was the relatively large variation between samples within the different categories of ovarian phenotypes (e.g. *GNRH1* for sub-region H1 and *KISS1* for sub-region H2).

Correlation of gene expression within hypothalamic sub-regions

Relative expression values for *GNRH1, KISS1, NPY, ESR1,* and *LEPR* were tested for correlations within sub-regions H1 and H2. Within sub-region H1, the highest correlation was between the relative expression of *ESR1* and *NPY* (Spearman's rank correlation coefficient value $\rho = 0.99$, p <0.01) (*Table 5*). Also within sub-region H1, *ESR1* and *NPY* expression were positively correlated with expression of *KISS1* ($\rho = 0.87$, p <0.01 and $\rho = 0.86$; p <0.01 respectively), *GNRH1* ($\rho = 0.81$, p <0.01) and *LEPR* ($\rho = 0.60$, p <0.05 and $\rho = 0.64$, p <0.05 respectively). The expression of *GNRH1* was positively

Table 3. Gene expression levels (mean ± SEM) in hypothalamic sub-regions H1 and H2 for suckled and weaned primiparous Brahman cows

Gene	Hypothalamic sub-region			
	H1		H2	
	Suckled (n = 6)	Weaned (n = 6)	Suckled (n = 5)	Weaned (n = 6)
GNRH1	502 ± 186a	198 ± 67a	98.9 ± 68.8a	10.5 ± 5.9a
KISS1	71.2 ± 24.1a	17.6 ± 9.6a	565 ± 200a	1318 ± 578a
ESR1	9.9 ± 1.9a	6.3 ± 1.4b	22.4 ± 8.5a	16.7 ± 3.6a
NPY	17.8 ± 3.3a	9.2 ± 1.1b	30.6 ± 6.7a	20.9 ± 4.4a
LEPR	90.2 ± 10.0a	43.9 ± 10.5b	222 ± 55a	106 ± 36a

Mean values with similar letters within rows and hypothalamic sub-region are not significantly different (p <0.05)

Table 4. Gene expression levels (mean ± SEM) in hypothalamic sub-regions H1 and H2 for primiparous Brahman cows with different ovarian phenotypes

Gene	Hypothalamic sub-region					
	H1			H2		
	Ovarian phenotype			Ovarian phenotype		
	OP1 (n = 4)	OP2 (n = 4)	OP3 (n = 4)	OP1 (n = 3)	OP2 (n = 4)	OP3 (n = 4)
GNRH1	247 ± 102a	562 ± 284a	241 ± 89a	3.2 ± 2.5a	127 ± 81a	9.6 ± 8.4a
KISS1	67.3 ± 37.9a	40.1 ± 22.6a	26.0 ± 12.7a	584 ± 208a	577 ± 290a	1668 ± 821a
ESR1	7.9 ± 1.9a	8.8 ± 3.2a	7.6 ± 1.6a	14.4 ± 06a	26.6 ± 10.7a	15.7 ± 4.4a
NPY	16.7 ± 4.3a	13.4 ± 4.7a	10.5 ± 0.8a	29.8 ± 6.8a	30.8 ± 8.2a	16.6 ± 3.9a
LEPR	99.3 ± 10.6a	59.2 ± 10.7b	42.6 ± 16.4b	224 ± 26a	176 ± 77a	92.4 ± 50.6a

OP1 = suppressed ovarian follicular growth; OP2 = active follicular growth without ovulation; OP3 = follicular growth with ovulation
Mean values with similar letters within rows, and hypothalamic sub-region, are not significantly different at $p < 0.05$

correlated with *KISS1* expression within sub-region H1 ($\rho = 0.67$, $p < 0.05$). Within sub-region H2, there were positive correlations between the expression of *ESR1* and *NPY* ($\rho = 0.99$), *ESR1* and *LEPR* ($\rho = 0.93$) and *LEPR* and *NPY* ($\rho = 0.91$).

Discussion

The present study demonstrates marked regional differences in the expression of *GNRH1* and *KISS1* within the hypothalamus of the bovine brain. The expression of KISS1 in the bovine brain is reported for the first time with the greatest expression observed in sub-region H2. This sub-region contains the posterior medial basal hypothalamus-median eminence, ventromedial hypothalamus, posterior portion of the arcuate nucleus, and the anterior portion of the mammillary body. The expression of *KISS1* in this region of the hypothalamus is consistent with predominant expression in the arcuate nucleus in sheep (Estrada et al. 2006; Smith et al. 2007; Smith 2009) and the anteroventral periventricular nucleus in other species (Han et al. 2005; Smith et al. 2006; Popa et al. 2008).

Previous GnRH localisation studies in bovine using immunocytochemistry (Leshin et al. 1988) and *in situ* hybridisation (Weesner et al. 1993) reported a concentration of GnRH neurons in the preoptic area. The finding in the present study of predominant *GNRH1* expression in the same area confirms the importance of this region of the hypothalamus for GnRH synthesis in bovine. The same region of the hypothalamus also has the highest *GNRH1* expression in sheep (Harris et al. 1998)) and rats (Jennes and Conn 1994).

Less marked regional expression was observed for *ESR1, LEPR* and *NPY,* and all three of these genes had greater expression in sub-region H2 than sub-region H1. The similar regional pattern of *ESR1, LEPR* and *NPY* expression is consistent with reports of estrogen and leptin receptors on *NPY* neurons (Williams et al. 1999; Tena-Sempere 2006; Acosta-Martinez et al. 2007; Smith 2009). It is also consistent with the coordinated actions of estrogen and leptin within the hypothalamus (Gao and Horvath 2008).

Receptors for estrogen and leptin have been reported on *KISS1* neurons (Williams

Table 5. Spearman's rank correlation coefficients (ρ) for the expression of *GNRH1, ESR1, NPY, LEPR* and *KISS1* within hypothalamic sub-regions H1 and H2 for primiparous Brahman cows

Gene	Hypothalamic sub-region (H1) Gene					Hypothalamic sub-region (H2) Gene				
	GNRH1	*ESR1*	*NPY*	*LEPR*	*KISS1*	*GNRH1*	*ESR1*	*NPY*	*LEPR*	*KISS1*
GNRH1		0.81**	0.81**	0.45	0.67*		–0.10	–0.04	–0.24	–0.34
ESR1	0.81**		0.99**	0.60*	0.87**	–0.10		0.99**	0.93**	0.46
NPY	0.81**	0.99**		0.64*	0.86**	–0.04	0.99**		0.91**	0.43
LEPR	0.45	0.60*	0.64*		0.52	–0.24	0.93**	0.91**		0.49
KISS1	0.67*	0.87**	0.86**	0.52		–0.34	0.46	0.43	0.49	

*(p <0.05); ** (p <0.01)

et al. 1999; Tena-Sempere 2006; Acosta-Martinez et al. 2007; Smith 2009) and, given that the greatest expression of *KISS1* was also in sub-region H2, the present findings strongly support morphological and functional relationships between *ESR1, LEPR* and *KISS1* in the bovine hypothalamus. The localisation of regional expression for *GNRH1, KISS1, ESR1, LEPR* and *NPY* in the bovine hypothalamus contributes important information for future studies on the regulation of these important reproductive genes in bovine.

The expression of *ESR1, LEPR* and *NPY* was lower in weaned cows compared with suckled cows for both hypothalamic sub-regions H1 and H2, although differences in expression for the three genes were only significant for sub-region H1. While our data do not explain the molecular or cellular basis for this coordinated change in gene expression, the fact that *ESR1, LEPR* and *NPY* were all expressed at a lower level in weaned cows is consistent with their above-mentioned interrelationships within the hypothalamus. There was a suggestion that the expression of *KISS1* in sub-region H2 was greater for weaned cows compared with suckled cows but this was not significant.

A feature of gene expression in the present study was the relatively large variation in expression for hypothalamic tissues in the same lactation and ovarian phenotype categories. For *GNRH1, KISS1* and *NPY,* the variation in expression within treatment categories might be explained, in part, by the pulsatile nature of their secretion but this would be considered less likely to explain the variation for *ESR1* and *LEPR*. Notwithstanding the individual variation in gene expression, it could be speculated that the apparent decrease in *ESR1* expression in weaned cows could be associated with a decrease in estrogen negative feedback on GnRH, and increased secretion of FSH and LH required for the resumption of increased follicular growth and ovulation which indeed occurred in weaned cows.

There were no apparent relationships between ovarian phenotype and gene expression, except for greater *LEPR* expression associated with ovarian phenotype 1 (follicles 4 – 5 mm) compared with ovarian phenotype 2 (follicles 10 mm) and ovarian phenotype 3 (ovulation), which did not differ. A relatively small number of cows were represented for the different ovarian phenotypes and, given the variation in gene expression highlighted above; further studies are required in postpartum cows to define relationships between phases of ovarian follicular transition and hypothalamic gene expression.

Conclusion

The present study provides fundamental information on regional expression of *GNRH1, KISS1, ESR1, LEPR* and *NPY* within the bovine hypothalamus. Preliminary information on interrelationships between the expressions of these genes is also provided. The ability to simultaneously

determine region specific expression of gene groupings within the hypothalamus provides a powerful approach to understanding complex gene networks linked to the effects of nutrition, lactation, ovarian steroids, metabolic homeostasis and maternal bonding on central mechanisms associated with postpartum reproduction in bovine.

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References

Acosta-Martinez, M., Horton, T. and Levine, J.E. (2007). Estrogen receptors in neuropeptide Y neurons: at the crossroads of feeding and reproduction. *Trends Endocrinol. Metab.* 18: 48 – 50

Daftary, S.S. and Gore, A.C. (2004). The hypothalamic insulin-like growth factor-1 receptor and its relationship to gonadotropin-releasing hormones neurones during postnatal development. *J. Neuroendocrinol* 16: 160 – 169

Diskin, M.G., Mackey, D.R., Roche, J.F. and Sreenan, J.M. (2003). Effects of nutrition and metabolic status on circulating hormones and ovarian follicle development in cattle. *Anim. Reprod. Sci* 78: 345 – 370

Estrada, K.M., Clay, C.M., Pompolo, S., Smith, J.T. and Clarke, I.J. (2006). Elevated KiSS-1 expression in the arcuate nucleus prior to the cyclic preovulatory gonadotrophin-releasing hormone/lutenising hormone surge in the ewe suggests a stimulatory role for kisspeptin in oestrogen-positive feedback. *J. Neuroendocrinol* 18: 806 – 809

Gamba, M. and Pralong, F.P. (2006). Control of GnRH neuronal activity by metabolic factors: the role of leptin and insulin. *Mol. Cell Endocrinol.* 254 – 255: 133 – 139

Gao, Q. and Horvath, T.L. (2008). Cross-talk between estrogen and leptin signaling in the hypothalamus. *Am. J. Physio.l Endocrinol. Metab.* 294: E817 – 826

Han, S.K., Gottsch, M.L., Lee, K.J., Popa, S.M., Smith, J.T., Jakawich, S.K., Clifton, D.K., Steiner, R.A. and Herbison, A.E. (2005). Activation of gonadotropin-releasing hormone neurons by kisspeptin as a neuroendocrine switch for the onset of puberty. *J. Neurosci.* 25: 11349 – 11356

Harris, T.G., Robinson, J.E., Evans, N.P., Skinner, D.C. and Herbison, A.E. (1998). Gonadotropin-releasing hormone messenger ribonucleic acid expression changes before the onset of the estradiol-induced luteinizing hormone surge in the ewe. *Endocrinology* 139: 57 – 64

Jennes, L. and Conn, P.M. (1994). Gonadotropin-releasing hormone and its receptors in rat brain. *Front Neuroendocrinol* 15: 51 – 77

Leshin, L.S., Rund, L.A., Crim, J.W. and Kiser, T.E. (1988). Immunocytochemical localization of luteinizing hormone-releasing hormone and proopiomelanocortin neurons within the preoptic area and hypothalamus of the bovine brain. *Biol. Reprod.* 39: 963 – 975

Lucy, M.C. (2003). Mechanisms linking nutrition and reproduction in postpartum cows. *Reprod. Suppl.* 61: 415 – 427

Messager, S., Chatzidaki, E.E., Ma, D., Hendrick, A.G., Zahn, D., Dixon, J., Thresher, R.R., Malinge, I., Lomet, D., Carlton, M.B., Colledge, W.H., Caraty, A. and Aparicio, S.A. (2005). Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54. *Proc. Natl. Acad. Sci. USA* 102: 1761 – 1766

Montiel, F. and Ahuja, C. (2005). Body condition and suckling as factors influencing the duration of postpartum anestrus in cattle: a review. *Anim. Reprod. Sci.* 85: 1 – 26

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29: 2003 – 2007

Popa, S.M., Clifton, D.K. and Steiner, R.A. (2008). The role of kisspeptins and GPR54 in the neuroendocrine regulation of reproduction. *Annu. Rev. Physiol.* 70: 213 – 238

Ramakers, C., Ruijter, J.M., Deprez, R.H. and Moorman, A.F. (2003). Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci. Lett.* 339: 62 – 66

Roberts, A.J., Klindt, J. and Jenkins, T.G. (2005). Effects of varying energy intake and sire breed on duration of postpartum anestrus, insulin like growth factor-1, and growth hormone in mature crossbred cows. *J. Anim. Sci.* 83: 1705 – 1714

Rozen, S. and Skaletsky, H. (2000). Primer3 on the WWW for general users and for biologist programmers. *Methods Mol. Biol.* 132: 365 – 386

Savio, J.D., Boland, M.P., Hynes, N. and Roche, J.F. (1990). Resumption of follicular activity in the early post-partum period of dairy cows. *J. Reprod. Fertil.* 88: 569 – 579

Short, R.E., Bellows, R.A., Staigmiller, R.B., Berardinelli, J.G. and Custer, E.E. (1990). Physiological mechanisms controlling anestrus and infertility in postpartum beef cattle. *J. Anim. Sci.* 68(3): 799 – 816

Shrestha, H.K., Nakao, T., Suzuki, T., Akita, M. and Higaki, T. (2005). Relationships between body condition score, body weight, and some nutritional parameters in plasma and resumption of ovarian cyclicity postpartum during pre-service period in high-producing dairy cows in a subtropical region in Japan. *Theriogenology* 64: 855 – 866

Silveira, P.A., Spoon, R.A., Ryan, D.P. and Williams, G.L. (1993). Evidence for maternal behavior as a requisite link in suckling-mediated anovulation in cows. *Biol. Reprod.* 49: 1338 – 1346

Smith, J.T. (2009). Sex steroid control of hypothalamic Kiss1 expression in sheep and rodents: Comparative aspects. *Peptides* 30: 94 – 102

Smith, J.T., Clay, C.M., Caraty, A. and Clarke, I.J. (2007). KiSS-1 messenger ribonucleic acid expression in the hypothalamus of the ewe is regulated by sex steroids and season. *Endocrinology* 148: 1150 – 1157

Smith, J.T., Clifton, D.K. and Steiner, R.A. (2006). Regulation of the neuroendocrine reproductive axis by kisspeptin-GPR54 signaling. *Reproduction* 131: 623 – 630

Team, R.D.C. (2006). *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing

Tena-Sempere, M. (2006). KiSS-1 and reproduction: focus on its role in the metabolic regulation of fertility. *Neuroendocrinology* 83: 275 – 281

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3: 1 – 12

Weesner, G.D., Harms, P.G., McArthur, N.H., Wilson, J.M., Forrest, D.W., Wu, T.J. and Pfaffl, D.W. (1993). Luteinizing hormone-releasing hormone gene expression in the bovine brain: anatomical localization and regulation by ovarian state. *Biol. Reprod.* 49: 431 – 436

Wettemann, R.P., Lents, C.A., Ciccioli, N.H., White, F.J. and Rubio, I. (2003). Nutritional- and suckling-mediated anovulation in beef cows. *J. Animal Sc.* 81: E48 – E59

Williams, L.M., Adam, C.L., Mercer, J.G., Moar, K.M., Slater, D., Hunter, L., Findlay, P.A. and Hoggard, N. (1999). Leptin receptor and neuropeptide Y gene expression in the sheep brain. *J. Neuroendocrinol.* 11: 165 – 169

Abstrak

Tujuan kajian ini adalah untuk memastikan ekspresi berbilang gen dalam hipotalamus lembu pedaging selepas beranak (postpartum) yang masih menyusukan anak atau mempunyai anak yang telah dicerai susu serta yang mempunyai fenotip ovari yang berlainan. Gen yang dikaji ialah gen hormon pelepas gonadotrofin (*GNRH1*), kisspeptin (*KISS1*), reseptor estrogen alpha (*ESR1*), reseptor leptin (*LEPR*) dan neuropeptida Y (*NPY*) dalam dua bahagian hipotalamus; H1 (anterior) dan H2 (ventral posterior). Ekspresi gen *GNRH1* dalam H1 adalah 7 kali ganda lebih besar ($p < 0.01$) daripada H2, manakala ekspresi gen *KISS1* dalam H2 adalah sekitar 20 kali ganda lebih besar ($p < 0.01$) daripada H1. Kedua-dua gen *ESR1* dan *NPY* menunjukkan ekspresi yang lebih besar ($p < 0.05$) dalam H2 daripada H1. Lembu yang dicerai susu mempunyai ekspresi gen *ESR1, LEPR* dan *NPY* lebih rendah ($p < 0.05$) pada bahagian H1 daripada lembu yang menyusu. Lembu dengan pertumbuhan folikel yang disekat (folikel 4 – 5 mm) mempunyai ekspresi gen *LEPR* yang lebih besar ($p < 0.05$) dalam H1 berbanding lembu dengan pertumbuhan folikel yang aktif (folikel >10 mm) dan lembu yang telah ovulasi. Ini merupakan laporan pertama ekspresi gen *KISS1* dalam otak lembu dan juga perbezaan bahagian dalam ekspresi berbilang gen dalam hipotalamus lembu. Kajian ekspresi gen secara serentak dalam hipotalamus menyediakan satu pendekatan baru dalam memahami rangkaian gen yang kompleks yang dikaitkan dengan kesan pemakanan, laktasi, steroid ovari, homeostasis metabolik dan hubungan ibu-anak ke atas mekanisma berpusat yang berkaitan dengan pembiakan lembu selepas beranak.

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