Weaning induced expression changes of genes associated with lactation and oestrogen signalling in the hypothalamus of postpartum cows

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Abstract

Weaning influences the hypothalamic control of reproduction. To understand how weaning affects hypothalamic gene expression patterns in beef cows, RNA samples from the anterior and ventral posterior hypothalamic regions of suckled and weaned primiparous cows were hybridized to Agilent bovine microarray to reveal possible interactions. In total, 199 differentially expressed genes were observed between suckled and weaned cows. Among these genes, gene ontology-molecular function terms *hormone activity* and *signal transducer activity* and KEGG pathway *neuroactive–ligand receptor interaction* were significantly over-represented as a response to weaning. Ten genes associated with physiological processes characteristic of lactation, namely osmolarity and stress, energy balance and suckling were revealed differentially expressed. These genes included angiotensin receptor 1 (*AGTR1*), arginine vasopressin (*AVP*), calcitonin-related polypeptide beta *(CALCB)*, corticotrophin releasing hormone binding protein *(CRHBP)*, neuropeptide Y *(NPY)*, growth hormone *(GH)*, growth hormone releasing hormone *(GHRH)*, agouti related protein homolog (*AGRP)*, oxytocin receptor (*OXTR)*, prolactin (*PRL)*. In addition, 37 genes encoded transcription factors, hormones and proteins that were either modulated by oestrogen or involved in oestrogen signaling in various tissues. *ESR1* and 9 of these genes had the same regional expression where eight of these genes coded for either a hormone or receptor. The significant differential expression of *AGRP*, *NPY*, *ESR1* and *PRL* that was observed with microarray showed the same trend when verified by qRT-PCR. In summary, the altered expression of genes associated with lactation and oestrogen signaling in the hypothalamus upon weaning could be important in the control of postpartum reproduction.

Keywords: Postpartum anestrus, GNRH , microarray, reproduction, gene expression

Introduction

The control of postpartum reproduction requires an integration of endocrine and metabolic signals which are linked to lactation, body energy reserves and responses to nutrition (Roche, 2006). Numerous signalling molecules and hormones are

involved in this coordinated regulation, thus unmasking the mechanisms behind this integration is a difficult task (Chagas, Bass, et al., 2007).

Lactation is one of the major factors responsible for controlling postpartum reproduction. Lactation suppresses fertility and prolongs the duration of anoestrus period in postpartum cows (Abeygunawardena and Dematawewa, 2004). Ovulation in postpartum beef cows can be induced by weaning where weaning reverses the suckling-mediated inhibition of gonadotropin releasing hormone (GnRH) release. Both base concentrations and amplitude of GnRH pulses and amplitude of GnRH pulses in cerebrospinal fluid of the third ventricle increased in response to weaning and this was associated with greater luteinizing hormone (LH) pulsatility and a shortened anoestrus period (Gazal, Leshim, et al., 1998; Hes, Lake, et al., 2005; Montiel and Ahuja, 2005).

Several mechanisms have been proposed to be involved in the central control of lactation on reproduction. The suckling mechanism which suppresses GnRH release could involve the endogenous opioid peptide, namely methionine-enkephalin, β-endorphin, dynorphin-A (Malven, Parfet, et al., 1986). Inhibition of GnRH release due to lactation is also influenced by other factors such as nutrition and energy balance. Postpartum lactating animals respond to the change in energy balance by activating the release of neuropeptides from propiomelanocortin / cocaine- and amphetamine-regulated transcript (POMC–CART) and agouti-related protein/ neuropeptide Y (AGRP-NPY) neurons to repress or stimulate appetite respectively (Che, Li et al., 1999; Adam, Archer, et al., 2002). It is possible that upon weaning, one or more appetite regulating neuropeptides work in concert to signal the body to increase energy input which in turn, allows ovarian cyclicity to resume.

The negative effects of lactation on GnRH release could also be due to the persisting oestrogen negative feedback in the anoestrous females (Yavas and Walton, 2000). Although studied extensively, there is no agreement on the exact mechanisms of oestradiol-mediated GnRH release. This is because oestradiol can have negative and positive feedback, there is more than one receptor involved in oestradiol signalling, and many neuronal intermediaries are implicated in oestradiol control of GnRH release. It is still unclear what neuronal signalling is involved in the different oestradiol feedback. While the role of ESR1 in GnRH regulation is clear, the mechanisms involving ESR1 in GnRH regulation is not. There have been contradicting views on the signalling pathway subsequent to ESR1 activation. Two different downstream signalling pathways have been proposed. ESR1 modulates target gene expression by either (1) binding to a specific oestrogen response element (ERE) located in the promoters of the target genes and interacts with a series of co-activators (Cowley, Hoare, et al. 1997) or (2) signalling through promoter elements other than ERE, by protein-protein interactions with transcriptional factors such as the GC-rich Sp1-binding site (Wang, Dong, et al. 1999).

The mechanisms mentioned in this section as well as others could be involved in the resumption of GnRH pulsatility post weaning , Traditional techniques have so far unable to identify how different mechanisms are synchronised to regulate GnRH release in the postpartum animals. This study utilized a robust high throughput gene expression approach to understand hypothalamic gene interactions that could be important in initiating postpartum cyclicity upon weaning in beef cows.

Materials and Methods

Animals and treatments

The study was approved by The University of Queensland Animal Ethics Committee (SAS/719/06/CRC). Primiparous Zebu cows (Brahman, *Bos indicus*) were maintained on pasture and standard husbandry except when required for experimentation. Six cows were weaned between Days 20 and 37 postpartum and slaughtered either 6 (n = 2) or 13 (n = 4) days after weaning. Six contemporary, lactating cows were slaughtered on the respective days (n = 6). Cows were fed to maintain a body condition score of 3.5-4.0 (Scale 1.0 to 5.0).

Brain sampling and processing

Cows were slaughtered using the non-penetrating captive bolt technique. 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 2 sub-regions classified as H1 and H2 (Figure 1). Each sub-section was cut to a depth of 3 to 4 mm. Regions and nuclei included in the H1 sample 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. The H2 sample included the medial basal hypothalamus-median eminence, ventromedial hypothalamus, posterior portion of the arcuate nucleus, and anterior part of the mamillary body.

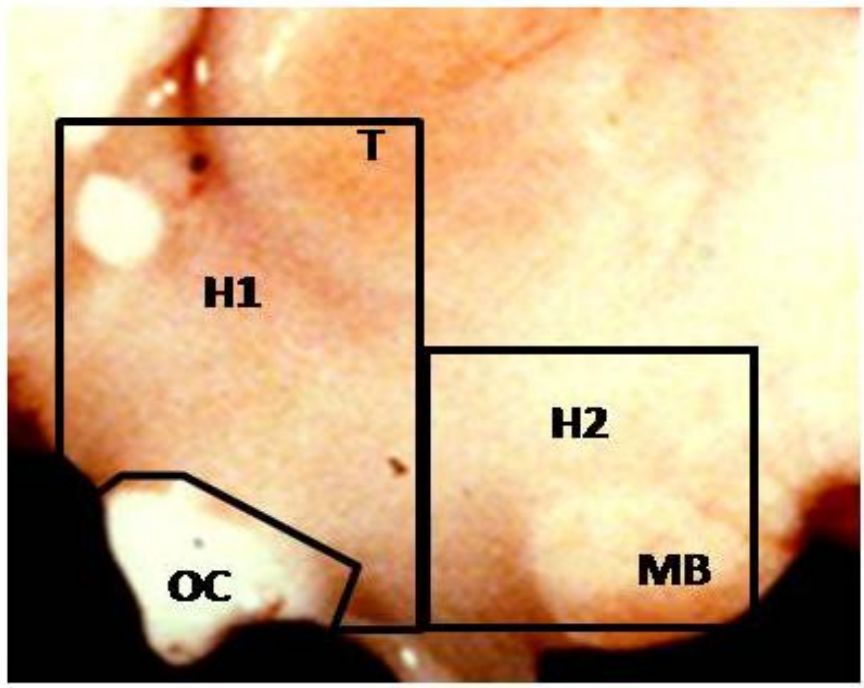

Figure 1. Hypothalamic sub-regions of the bovine brain.
The general sub-regions were: H1, anterior hypothalamus; H2, ventral posterior hypothalamus; Hypothalamic nuclei relevant to reproduction include: POA, preoptic area; AVPV, anteroventral periventricular nucleus; ARC, arcuate nucleus. Details of other hypothalamic nuclei contained in the sub-regions are provided in the text. MB, mammillary body; OC, optic chiasma; T, thalamus

Within 90 min, approximately 0.4 g of brain sample was submerged in 4 ml 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 RNA extraction.

RNA extraction and reverse transcription

Total RNA was extracted using modified RNeasy extraction kit (Qiagen) protocol. 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). cDNA was synthesized using standard Superscript III Reverse Transcriptase protocol (Invitrogen ,USA) 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 electrophoresis.

Linear amplification of RNA

For microarray experiment, an aliquot of 500 ng of each RNA sample was reverse-transcribed. Samples were amplified and either labelled with Cyanine 3 (Cy3) or Cyanine 5 (Cy5) by T7-polymerase in vitro transcription, to give fluorescent-labelled cRNA using the low RNA input Linear Amplification Kit from Agilent Technologies (Forest Hills, Victoria, Australia).

Microarray platform

The Agilent Bovine-Four-Plex G2519f DNA oligonucleotide microarray (Agilent Technologies) utilised contained 21,475 unique 60-mer probes representing approximately 19,500 distinct bovine genes. The sequences and annotations of the probes are available at http://www.chem.agilent.com/. The functional annotations of the probes were derived from their human orthologs. Approximately 5,107 (24%) probes on the microarray are not linked to a reference sequence (RefSeq) and thus, the identity and function is unknown. To reveal identities of more probes, all the Agilent probes were reannotated as follows: 1) All probes were annotated with direct BLAST searches (http://www.ncbi.nlm.nih.gov/) and 2) All probes were aligned to the human and bovine Refseq collection mapped on the bovine genome assembly (Btau 4.0). 3) All probes were further tested for overlaps or close proximity to bovine RefSeq. These procedures identified 2,889 previously unknown sequences and 1,007 probes were flagged as potential new annotations as they met some but not all of the criteria above.

Microarray hybridisation

Microarrays were hybridised according to standard protocol at the SRC Microarray Facility of the Institute for Molecular Biosciences in Brisbane Australia (http://microarray.imb.uq.edu.au).
Hybridisation was conducted in an Agilent DNA Microarray Hybridisation Oven using Gene Expression Hybridisation Kit and Wash Buffer Kits from Agilent Technologies according to the manufacturer's instructions. In general, 825ng each of the Cy3 and Cy5 labelled complementary RNA (cRNA), 11 µl 10X blocking agent and 2.2 µl 25X fragmentation buffer were incubated at 60°C for 30 min to fragment the RNA. Then, hybridisation was performed with 55 µl 2X GEx Hybridisation Buffer H1-RPM. Hybridisation mix was dispensed in the Agilent SureHyb chamber and placed in rotisserie in a hybridisation oven at 65°C for 17 h. The hybridised slides are washed in pre-warmed Gene Expression Wash Buffer 1 and 2 for 1 min each.

Microarray scanning and spot quantification

Arrays were placed in Agilent Stabilisation and Drying Solution prior to scanning on an Agilent G2565AA scanner. Raw data processing was performed using Agilent Feature Extraction Software. To ensure the quality of image analysis, several quality control measures were performed including spot quality, normalisation against background, reproducibility, uniformity and sensitivity.

Data normalisation and identification of significantly changed genes

Data normalisation was achieved using mixed model analysis which is regarded as the most optimal method (Reverter et al., 2005). Differentially expressed genes were identified by model-based clustering by a mixture of distributions on the normalised expression of each gene at each cross and time point as previously detailed (Reverter et al., 2004; Reverter et al., 2005). The mixed model was fitted to the intensity readings as below:

$$Y_{ijkhgn} = \mu + C_{ijk} + G_g + AG_{ijg} + DG_{kg} + VG_{hg} + \varepsilon_{ijkhgn} \quad (1)$$

where $Y_{ijkhsmn}$ represents the n^{th} background-adjusted, normalised base-2 log-intensity from the g^{th} gene (or probe) at the h^{th} phenotype variety (treatment and hypothalamus region), from the i^{th} chip, j^{th} array (i.e., there are four microarrays per chip) and k^{th} dye channel; μ is the overall mean; C represents a comparison fixed group effects with 64 levels and defined as those intensity measurements from the same chip, array and dye channel; G represents the random gene (or probe) effects with 21,475 levels; AG, DG, and VG are the random interaction effects of array by gene, dye by gene, and variety by gene, respectively; and finally, ε is the random error term. . For the random effects in Model (1), standard stochastic assumptions are:

$G \sim$ iid $N(0, \sigma^2_g)$,
$AG \sim$ iid $N(0, \sigma^2_{ag})$,
$DG \sim$ iid $N(0, \sigma^2_{dg})$,
$VG \sim$ iid $N(0, \sigma^2_{hg})$,
and $\varepsilon \sim$ iid $N(0, \sigma^2_e)$,

where iid denotes independently and identically distributed and N denotes the normal distribution. Variance components are between genes (σ^2_g), between genes within array (σ^2_{ag}), between genes within dye (σ^2_{dg}), between genes within phenotype (σ^2_{hg}), and within genes (σ^2_e). Variance components were estimated using restricted (to zero error contrasts) maximum likelihood [(REML;,(Searle, Casella et al., 1992) for detailed formulae].

Table 1. Calculations for differential expression *(DE)* in the treatment analysis

Regions	H1,H2 and H1H2
Differential expression *DE* determined	$\boldsymbol{DE^{H1}_{g}} = VG_{gw} - VG_{gs}$ (within H1) (1) $\boldsymbol{DE^{H2}_{g}} = VG_{gw} - VG_{gs}$ (within H2) (2) $\boldsymbol{DE^{H1H2}_{g}} = [ave\ (VG^{H1}, VG^{H2})]_{gw} - ave\ (VG^{H1}, VG^{H2})]_{gs}$ (combined H1 and H2) (3)

Finally, the differentially expressed measurement contrasts in (1) to (3) were processed by fitting a two-component normal mixture model and posterior probabilities of belonging to the non-null component were used to identify differentially expressed genes with an estimated experiment-wise false discovery rate of < 1% as previously described (McLachlan et al., 2006). The microarray data are publicly accessible in MIAME format at the GEO database (http://www.ncbi.nlm.nih.gov/geo/).

Gene annotations and functional enrichment clustering

All gene annotations, functional enrichment clustering, and mapping to existent functional pathways, utilised the DAVID software (http://david.abcc.ncifcrf.gov/). Differentially expressed genes were analysed for gene annotation and functional enrichment clustering within H1 and H2 and normalised expression values of the two regions were averaged prior to determination of fold change (H1H2). Analysis was done against a background list of bovine sequences represented by probes on the bovine Agilent array. Only probes associated with a Refseq are used as background genes and are listed in http://www.chem.agilent.com/en-US/products/instruments/dnamicroarrays/pages/gp58802.aspx.database (NCBI http://www.ncbi. nlm.nih.gov/:Accessed January 2008).

Determination of gene function

The function of each gene revealed differentially expressed was determined using literature search. Differentially expressed genes were annotated using the Database for Annotation and Visualisation and Integrated Discovery (DAVID) software. Further literature searches on the function of each of the genes was undertaken as follows: (1) official gene nomenclature was obtained at the genecards website (http://www.genecards.org/), (2) the gene symbol, official or alternate names as well as lactation, hypothalamus, oestrogen, GnRH, energy balance, were used as keywords for literature searches and (3) genes with similar function were then grouped together based on the expression patterns. The specificity of each probe was verified using BLAST or alignment with bovine NCBI Reference Sequence Number (Refseq) mapped on the bovine genome. Only genes that aligned to the exon of one bovine Refseq or to Refseq of more than one species were described.

Quantitative Real Time PCR (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 2. 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. Reactions were 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^{o}C for 2 min; Step 2, 95^{o}C for 10 min; Step 3, 95^{o}C for 15 sec and 60^{o}C for 1 min. Step 3 was repeated for 40 cycles. A melt curve analysis step was included (95^{o}C for 15 sec, 60^{o}C for 15 sec and 95^{o}C for 15 sec) to confirm the specificity of the reaction. Furthermore, PCR products were separated on agarose gel and

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

Normalisation of PCR data

Genes were normalised against tryptophan monooxygenase activation protein, zeta polypeptide (*YWHAZ*) which was found to be stably expressed across all cows 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 according to 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). Hypothalamic sub-regions, treatment, day of collection, and days postpartum, were fitted as fixed variables in a linear model before factorial ANOVA. Significance was set at $P < 0.05$. The differences between means for the factorial levels were tested by one-way ANOVA.

Table 2. Primers used for real time RT-PCR

Gene		Forward primer (5'-3')	Reverse Primer (5'-3')
ESR1	Estrogen receptor α	ATGATGAAAGGCGGAA TACG	AAGGTTGGCAGCTCTC ATGT
NPY	Neuropeptide Y	CAGGCAGAGATACGGG AAAC	GGGAGGACTGGCAGAC TCTA
PRL	Prolactin	ACCCTGTGTGGTCAGGA CTC	TGTGGGCTTAGCAGTTG TTG
AGRP	Agouti related protein homolog	GCCTGAGGAAGCCTTAT TCC	GCAGAAGGCGTTGAAG AAAC
PNRC2	Proline-rich nuclear receptor coactivator 2	ATCGGCCCATGAAAACT TCT	TCTCTCCACCACCCATC TTC
YWHAZ	Tyrosine3-monooxygenase /tryptophan monooxygenase activation protein, zeta polypeptide	ACCTACTCCGGACACAG AACAT	GAAGATTCCTCTCCTCA TTGGA

Results and Discussion

Effects of weaning on ovarian activity

Cows that continued to suckle a calf typically had ovaries with suppressed follicular growth except for two suckled cows that showed evidence of resumption of follicular growth. The latter two cows were

at postpartum Day 51 and 57 (Table 3). The ovaries of cows weaned 6 d before slaughter had growing follicles (10 mm diameter) whilst cows weaned 13 d before slaughter all showed evidence of recent ovulations (corpus luteum or corpus hemorrhagicum) (Table 3). It can be concluded that cows weaned at 20 to 36 d postpartum resumed ovarian activity within a few days of weaning and ovulation occurred between 7 and 13 d after weaning.

Differential gene expression in suckled and weaned cows

The two comparisons performed compared hypothalamic sub-regions H1 and H2 and suckled and weaned. The number of differentially expressed genes in the H1-H2 comparison was greater than the suckled-weaned comparison. For suckled cows, there were 347 differentially expressed genes between H1 and H2, and for weaned cows there were 239 differentially expressed genes between H1 and H2. For the suckled-weaned comparison, there were 122 differentially expressed genes in H1 and 84 differentially expressed genes in H2. When normalised expression values of the two sub-regions were averaged before determination of fold change (H1H2) there were 152 differentially expressed genes between suckled and weaned cows. In total, upon weaning, there were a total of 199 differentially expressed genes (Figure 2).

Table 3. Effect of weaning and postpartum period on ovarian activity in primiparous Brahman cows

Anim. no.	Treatment	Days after weaning at slaughter	Days postpartum at slaughter	Ovarian activities
1	Suckled (control)	1	32	Suppressed ovaries (OP1)
2	Suckled (control)	1	30	Suppressed ovaries (OP1)
3	Suckled (control)	1	38	Suppressed ovaries (OP1)
4	Suckled (control)	1	39	Suppressed ovaries (OP1)
5	Suckled (control)	1	57	Ovaries approaching ovulation (OP2)
6	Suckled (control)	1	51	Ovaries approaching ovulation (OP2)
7	Weaned	6	28	Ovaries approaching ovulation (OP2)
8	Weaned	6	26	Ovaries approaching ovulation (OP2)
9	Weaned	13	33	Ovulated ovaries (OP3)
10	Weaned	13	35	Ovulated ovaries (OP3)
11	Weaned	13	46	Ovulated ovaries (OP3)
12	Weaned	13	50	Ovulated ovaries (OP3)

Gene annotation and functional enrichment clustering

For H1, 76 of 122 differentially expressed genes submitted as a gene list were annotated. For H2, 69 of 84 differentially expressed genes were annotated, and for

H1H2 103 of 152 differentially expressed genes were annotated. Of the 18,519 Agilent probes submitted as a background, 16,796 were utilised with successful annotation by DAVID software.

The functional theme that emerged as over-represented for differentially expressed genes within H2 and H1H2 was the same and consisted of the gene ontology-molecular function terms *hormone activity* and *signal transducer activity*. The common differentially expressed genes between H2 and H1H2 associated with both of the terms were *AVP, CGA, NPY, PRL, GH* and *GHRH*. A slightly different functional theme was observed for H1 where only gene ontology-molecular function terms *cation binding* was over-represented.

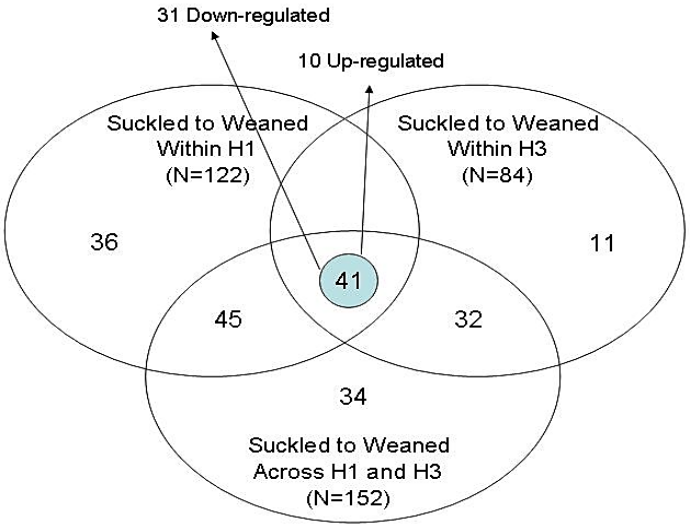

Figure 2. Venn diagram representing the distribution of differentially expressed probes across the anterior hypothalamic region H1, posterior hypothalamic region H2 and combined region H1H2 in the treatment analysis.

Genes associated with lactation

The list of genes differentially expressed between suckled and weaned cows included 10 genes that are associated with physiological processes characteristic of lactation. Evidence of relationship between genes or their encoded protein with energy balance, milk synthesis, suckling or fluid homeostasis were used as criteria for the association with lactation. Two differentially expressed genes implicated in osmolarity and stress, angiotensin receptor 1 (*AGTR1*) and arginine vasopressin (*AVP*), had greater expression in weaned than suckled cows (Table 4). Genes associated with energy balance such as *NPY, GH,* and *GHRH* were DE in H2 and H1H2. *AGRP* was differentially expressed in all regions (Table 4). Three genes associated with suckling *OXTR, PRL* and a regulator of WAP, *NFIA*, had greater expression in weaned cows. *OXTR* mRNA was differentially expressed only in H1 while *PRL* was differentially expressed in H1, H2 and H1H2 (Table 4).

The expression of two other genes associated with stress, calcitonin-related polypeptide beta (*CALCB)* and corticotrophin

releasing hormone binding protein *(CRHBP)* had greater expression in H2 and H1, respectively, in suckled cows.

Genes regulated by estrogen or associated with oestrogen signaling

Of 199 differentially expressed genes annotated, 37 genes encode transcription factors, hormones and proteins that are either modulated by oestrogen or involved in oestrogen signalling in various tissues (Appendix A). *ESR1* was differentially expressed in H2 and H1H2 and 9 genes had the same regional expression as *ESR1*.

Table 4. Differentially expressed genes related to lactation that were expressed at a (A) higher and (B) lower level in the hypothalamus of weaned when compared to suckled primiparous Brahman cows

Gene name	H1 (n=6)	H2 (n=6)	H1H2 (n=6)
(A)			
Angiotensin receptor 1 *(AGTR1)*	1.00	4.79	2.35
Arginine vasopressin *(AVP)*	1.00	3.40	2.23
(B)			
Agouti related protein homolog *(AGRP)*	0.49	0.21	0.32
Prolactin *(PRL)*	0.47	0.23	0.33
Growth hormone (GH)	1.00	0.36	0.53
Growth hormone releasing hormone *(GHRH)*	1.00	0.35	0.54
Neuropeptide Y *(NPY)*	1.00	0.34	0.58
Oxytocin receptor *(OXTR)*	0.64	1.00	1.00
Calcitonin-related polypeptide beta *(CALCB)*	1.00	0.64	1.00
Corticotropin releasing hormone binding protein *(CRHBP)*	0.74	1.00	1.00

Values are fold change within the anterior (H1) and ventral posterior (H2) hypothalamic sub-regions as well as combined regions H1H2 between suckled and weaned animals. Fold change value for each gene is the normalised intensity values of weaned animals divided by normalised intensity values of suckled animals. Values >1 indicates weaned>suckled. Values <1 indicates weaned<suckled. '1' indicates no fold change detected.

Eight of these genes code for either a hormone or receptor: *GH, GHRH, NPY, CALCB, GABRR2, AVP* and *AGTR1*. *GABRR2, AVP* and *AGTR1* had greater expression in weaned cows (Table 5) while *GH, GHRH, NPY* and *CALCB* had lesser expression in weaned cows (Table 6).

Three differentially expressed genes associated with oestrogen were part of the Wnt signalling pathway and were expressed exclusively in H1. Expression of WNT1 inducible signalling pathway protein 2 *(WISP2)* and R-spondin homolog *(RSPO1)* had lesser expression after weaning while expression of naked cuticle homolog 1 (*NKD1)* was greater after weaning. Two transcription factors associated with *ESR1* signalling, WWW domain binding protein (*WBP2)* and proline-rich nuclear receptor co activator (*PNRC2)* were differentially expressed. *WBP2* had higher expression in the combined regions H1H2 for weaned

cows (Table 5) while *PNRC2* had lower expression in H1, H2 and H1H2 of weaned cows when compared to suckled cows (Table 6). A component of the inactivated steroid receptor complex, peptidylprolyl isomerase D (*PPID*) had lesser expression in weaned cows (Table 6).

Comparison of microarray and qRT-PCR for suckled and weaned cows

The significant differential expression of *AGRP, NPY, ESR1* and *PRL* that was observed with microarray showed the same trend by qRT-PCR (Figure 3). Similar to the microarray findings, the expression of *PRL* and *AGRP* in H1 and H2 was less for weaned cows whilst the expression of *NPY* and *ESR1* in H2 was also less in weaned cows. The expression of *PNRC2* was less for weaned cows in H1 and H2 by microarray but this was only observed for H2 and not H1 by qRT-PCR.

Table 5. Hypothalamic genes related to oestrogen signalling or regulated by oestrogen that showed higher expression in weaned primiparous Brahman cows

Gene name	H1 (n=6)	H2 (n=6)	H1H2 (n=6)
Angiotensin receptor 1 *(AGTR1)*	1.00	4.79	2.35
Calcium channel, voltage-dependent, L type, alpha 1B subunit *(CACNA1B)*	2.04	4.29	2.97
PHD finger protein12 *(PHF12)*	1.00	3.90	2.61
Gamma-aminobutyric acid type B receptor *(GABBR2)*	1.00	3.55	2.67
Arginine vasopressin *(AVP)*	1.00	3.40	2.23
Carboxypeptidase M precursor *(CPM)*	1.96	3.24	1.00
WW domain binding protein 2 *(WBP2)*	1.00	1.00	2.19
Alcohol dehydrogenase 6 *(ADH6)*	2.02	1.00	2.44
Naked Cuticle homolog 1*(NKD1)*	2.12	1.00	1.00

Values are fold change within the anterior (H1) and ventral posterior (H2) hypothalamic sub-regions as well as combined regions H1H2 between suckled and weaned animals. Fold change value for each gene is the normalised intensity values of weaned animals divided by normalised intensity values of suckled animals. Values >1 indicates up-regulation (ie: weaned>suckled). '1' indicates no fold change detected.

Table 6. Hypothalamic genes related to oestrogen signalling or regulated by oestrogen that had higher expression in suckled primiparous Brahman cows

Gene name	H1 (n=6)	H2 (n=6)	H1H2 (n=6)
Agouti related protein homolog *(AGRP)*	0.49	0.21	0.32
Prolactin *(PRL)*	0.47	0.23	0.33
Glycoprotein hormones, alpha polypeptide *(CGA)*	0.64	0.26	0.41
Growth hormone *(GH)*	1.00	0.36	0.53
Growth hormone releasing hormone *(GHRH)*	1.00	0.35	0.54
Neuropeptide Y *(NPY)*	1.00	0.34	0.57
Nudix (nucleoside diphosphate linked moiety X)-type motif 1 *(NUDT1)*	0.50	0.43	0.46
Proline-rich nuclear receptor coactivator 2 *(PNRC2)*	0.55	0.70	0.62
Oestrogen receptor *(ESR1)*	1.00	0.64	0.73
Peptidylprolyl isomerase D (cyclophilin D) *(PPID)*	0.65	1.00	0.75
Calcitonin-related polypeptide 3 *(CALCB)*	1.00	0.64	0.78
Oxytocin receptor *(OXTR)*	0.64	1.00	1.00
Cytochrome P450, family 4, subfamily B, polypeptide 1 *(CYP4B1)*	0.69	1.00	1.00

Values are fold change within the anterior (H1) and ventral posterior (H2) hypothalamic sub-regions as well as combined regions H1H2 between suckled and weaned animals. Fold change value for each gene is the normalised intensity values of weaned animals divided by normalised intensity values of suckled animals Values <1 indicates weaned<suckled. '1' indicates no fold change detected.

PRL: prolactin; *AGRP*: agouti-related peptide; NPY: neuropeptide Y; *ESR1*: oestrogen-receptor alpha and *PNRC2*: proline-nuclear receptor co-activator 2. Y axis is log normalised mean expression. S: suckled; W: weaned. * $p<0.05$

Weaning removes the suckling stimulus, as well as visual and auditory stimuli provided by the presence of a calf. It also removes the nutritional drain of lactation, promotes the return to positive energy balance and promotes the resumption of ovulation in postpartum cows. Weaning was associated with the differential expression of 199 genes in the two hypothalamic sub-regions H1 and H2 and the combined sub-regions H1H2. Functional cluster analysis was performed separately for H1, H2 and combined regions H1H2. For regions H2 and H1H2, functional clusters identified to be significantly over-represented in the list of differentially expressed genes consisted of groups of genes associated with the molecular functions binding and signalling and, at a more specific level, hormone activity. Genes grouped under the term hormone activity were related to lactation which would be consistent with changes induced by weaning.

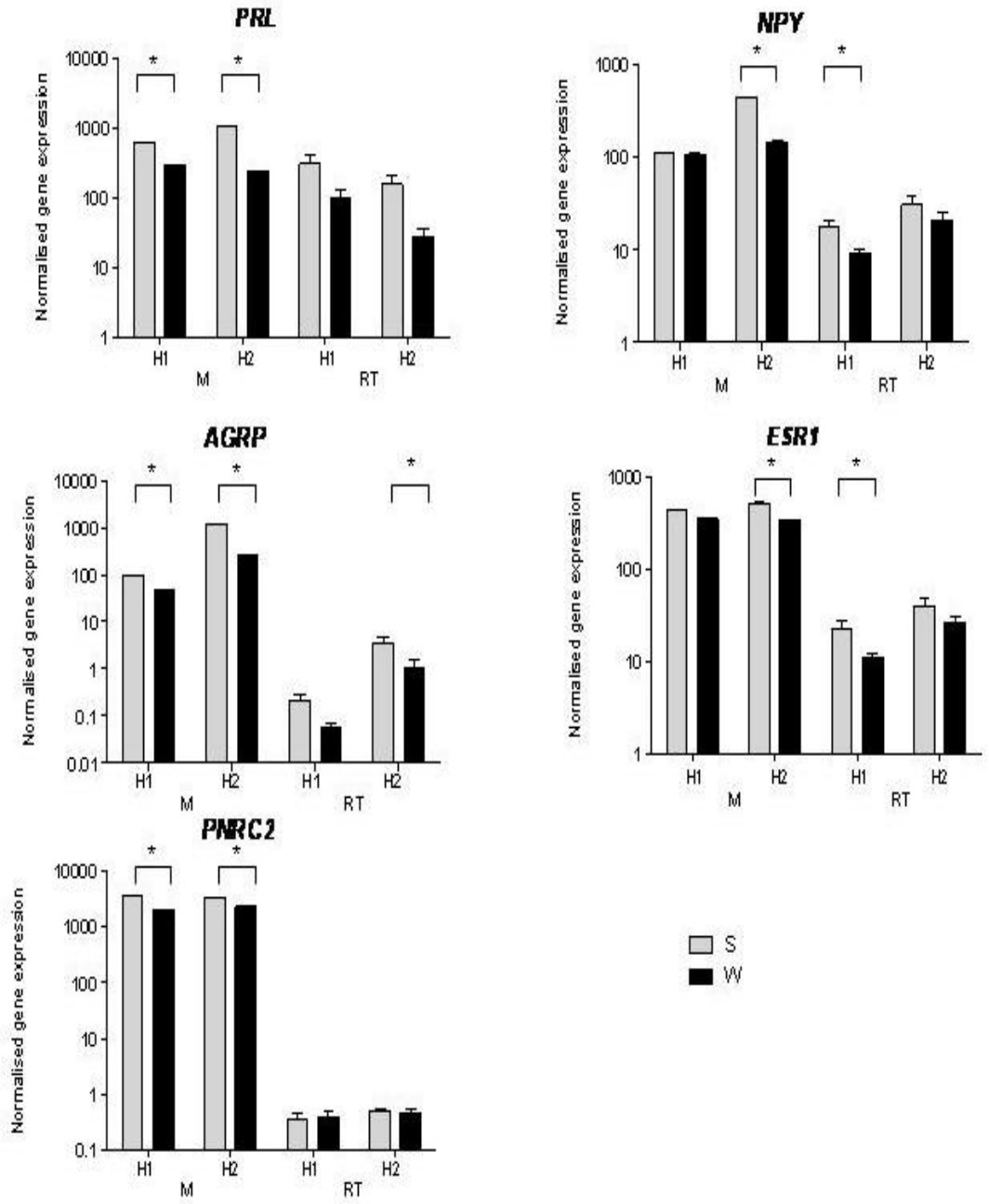

Figure 3. Gene expression patterns of genes differentially expressed between suckled and weaned animals within two hypothalamic sub-regions; anterior (H1) and ventral posterior (H2) as revealed in the microarray (M) and verified with qRT-PCR (RT) experiment

Hypothalamic genes associated with lactation were differentially expressed upon weaning which is a good confirmation of the model used. Genes associated with appetite regulation and body fluid homeostasis were differentially-expressed. The expression of

two genes associated with appetite regulation, *AGRP* and *NPY*, had lower expression in weaned cows for H2 and H1H2 (Richard, 2007) . These regions contained the ARC which is important for the regulation of energy balance and *NPY* and *AGRP* neurons were previously reported to be localised in the ARC (Smith and Grove, 2002; Crown, Clifton, et al., 2007). Two genes involved in the appetite suppression, proopiomelanocortin (*POMC*) and cocaine- and amphetamine-related peptide (*CART*) were not differentially expressed between suckling and weaned cows. A preliminary conclusion from these findings could be that any changes in appetite in response to weaning are associated with changes in appetite stimulating genes rather than appetite suppressing genes.

Another gene associated with lactation that was differentially expressed is prolactin *(PRL).* This is the first report of *PRL* expression in the bovine hypothalamus. Upon weaning, *PRL* mRNA was expressed at relatively high levels and expression was lower for weaned cows in H1, H2 and H1H2. The higher *PRL* expression in suckled animals in this study is consistent with the increased circulating prolactin level reported in lactating humans (Grattan, 2001). The reduction of *PRL* as well as *NPY, CRHBP* and *OXTR* expression in weaned cows is consistent with several functions that have been attributed to brain prolactin mainly based on the distribution of prolactin receptors (Quennell, Mulligan et al., 2009). Hyperprolactinaemia stimulates food intake, blocks stress-induced CRH and is associated with oxytocin release (Carter and Lightman, 1987; Koike, Miyake, et al., 1991; Noel and Woodside, 1993; Carter, Altemus, et al., 2001; Donner, Bredewold, et al., 2007). Given the similar lower expression of the above-mentioned genes in weaned cows, it could be hypothesized that prolactin, NPY, oxytocin and CRH are interrelated and/or share common regulatory mechanisms to adapt to physiological changes associated with the weaning of calf postpartum.

Oestrogen may play a role in linking lactation to reproduction in postpartum cows. *NPY, PRL, AGRP, OXTR, GHRH* and *AVP* which are regulators of GnRH as well as differentially expressed between suckled and weaned cows are regulated by oestrogen (Childs, Iruthayanathan, et al., 2005; Somponpun, 2007; Santollo and Eckel, 2008; Weiser, Foradori, et al. 2008). In addition, at least 37 other genes that were either associated with oestrogen signalling or were regulated by oestrogen are also differentially expressed between the hypothalamus of suckled and weaned cows. For the first time, the expression pattern of *ESR1* in response to weaning in postpartum cows was observed. The expression of *ESR1* was lower for weaned cows, in H2 and H1H2. The change of *ESR1* expression was in the sub-regions H2 and H1H2 which contained the ARC and VMH, nuclei that are known to include neurons that are responsive to oestradiol (Clarke, Pompolo, et al., 2001). Oestrogen negative feedback persists in anoestrous females even though circulating concentrations of oestrogen are low (Yavas and Walton, 2000). The changed expression of *ESR1* in response to weaning could be associated with reduced oestrogen negative feedback in the postpartum period of beef cows. As *ESR1* is important in reproduction, the decreased level of *ESR1* upon weaning could also play a role in the resumption of ovulation postpartum via regulation of GnRH release.

Additionally, several co-regulators and genes associated with oestrogen signalling involving ESR1 were also identified in the present study. These included, peptidylprolyl isomerase D (cyclophilin D) (*PPID*) and proline-rich nuclear receptor coactivator 2 (*PNRC2*). PPID is associated with inactivated steroid receptor including ESR1.

It is regulated by oestradiol and it transports ESR1 to the nucleus (Kumar, Mark, et al., 2001). Upon weaning, the similar expression of *PPID* and *ESR1* could indicate a possible function of PPID in the signal transduction process of *ESR1* that could contribute to the reduced oestrogen negative feedback upon weaning. Similarly, a co-activator of *ESR1*, *PNRC2* also had similar expression changes with *ESR1*. *PNRC2* influences the expression of target genes after the activation of ESR1 and the similar expression of *ESR1, PPID* and *PNRC2* may suggest a co-regulatory mechanism to respond to the increased oestrogen level upon weaning (Zhou and Chen, 2001; Zhou, Ye, et al., 2006). The findings for *PNRC2* and *PPID* suggested that a range of genes that require activation of ESR1 by oestrogen undergo changes in expression in the postpartum cow upon weaning. There is a possibility that the changes observed in the above-mentioned genes could also be involved in the modulation of oestrogen on the resumption of ovarian cyclicity postpartum.

Weaned cows had higher gene expression of arginine vasopressin (*AVP*) and angiotensin receptor 1 (*AGTR1*); genes that are associated with body fluid homeostasis (McKinley, Allen, et al., 2001; Somponpun, 2007). Administration of angiotensin stimulates water intake and the release of AVP in rhesus monkey (Simonnet, Rodriguez, et al., 1979). AGTR1 are localised throughout the brain including the POA and ME although AGTR1 has not been implicated with angiotensin regulation of AVP in mice (McKinley, Allen, et al., 2001). Thus, the similar expression pattern of *AGTR1* and *AVP* between the suckled and weaned cows could suggest a species-specific inter-relationship between angiotensin and vasopressin system in postpartum cows. Fluid balance in lactating animals is perturbed by the demand of milk ejection but the loss of water that accompanies milk transfer and vasopressin cell activation (Somponpun, 2007). The suckled animals were weaned 24 to 48 h prior to slaughter had lower *AVP* expression. Perhaps, the immediate response to weaning prior to slaughter could explain the lower level of *AVP* and *AGTR1* in the suckled cows when compared to weaned cows.

Although the analysis reported focused on gene expression changes as a response to weaning, the relationship between lactation and the regulation of reproduction via the control of GnRH was evident. Genes that were involved with lactation such as *NPY, PRL, AGRP, OXTR,GHRH* and *AVP* were mapped to the over-represented functional pathway : *neuroactive-ligand receptor interaction* and were linked to the regulation of GnRH (Herbison, 2006). Among the above-mentioned genes, *OXTR* could be an important link between lactation and the control of reproduction. Weaning reduced the lower expression of oxytocin receptor (*OXTR*). Oxytocin role during lactation is unequivocal. Oxytocin is associated with milk synthesis, fluid balance, stress as well as maternal behaviour (Burbach, Young, et al., 2006). It would be impossible to decipher the role of *OXTR* using the gene expression pattern revealed in postpartum cows. However, the expression of *OXTR* is differentially expressed in the sub-region H1 which would have contained the main population of GnRH neurons. It would be tempting to assume that *OXTR* is involved in the collective effects of different factors of lactation on the suppression of reproduction. Indeed, in rats, oxytocin receptors were reported on a sub-population of GnRH neurons and in GnRH explants, oxytocin stimulates GnRH release (Caligioni, Oliver, et al., 2007).

Four out of five genes differentially expressed between weaned and suckled cows tested by qRT-PCR were similar to the microarray results. In comparison, the

percentage of similar expression patterns revealed in the present study exceeds the percentage revealed in a relatively large study in mice where around 73% of genes showed similar expression patterns with microarray and qRT-PCR (Morey, Ryan, et al., 2006). Less significant changes are observed with qRT-PCR than with microarray experiment. This could be due to differences in the technical aspects of the two experiments or the statistical analysis of both methods. The normalisation of qRT-PCR results to fewer genes compared to microarray experiments could cause qRT-PCR results to be more susceptible to systemic variations. Nevertheless, similar trends between qRT-PCR and microarray experiments are considered successful comparison (Morey, Ryan, et al., 2006).

Genes with relatively low fold changes in expression were not consistently identified as differentially expressed by microarray and qRT-PCR (for example: *PNRC2*). The inability to compare microarray experimental data with other gene expression techniques for genes with low fold changes has been reported (Wurmbach, Yuen, et al., 2003). However, the microarray platform utilised in the present study has robust methodology, a high level of control and stringent statistical analysis (Hughes, 2006). Together with identification of over-represented functional clusters in the differentially expressed genes, that are relevant to both lactation and reproduction, the results for the microarray experiments are therefore considered to be indicative of the biology of the experimental animals.

Although a small number of cows were used in this study and a relatively small number of genes were used to compare gene expression patterns obtained with microarray and qRT-PCR, the combined microarray and qRT-PCR findings provided strong evidence that the changes in gene expression associated with lactation status were reflective of underlying biological processes.

Conclusion

Utilization of micro-array technology reveals groups of differentially expressed genes that are functionally clustered or had similar pattern in bovine hypothalamus upon weaning. Similarity in gene expression patterns can be used as an indicator of similar co-regulatory or gene regulation mechanisms. Although further protein studies are needed to confirm this notion, results obtained in this study can be used as a starting point towards understanding the intricate integrated mechanism of lactational control of reproduction.

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