# The bovine mammary gland expresses multiple functional isoforms of serotonin receptors

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## Abstract

Recent studies in dairy cows have demonstrated that serotonergic ligands affect milk yield and composition. Correspondingly, serotonin (5-HT) has been demonstrated to be an important local regulator of lactational homeostasis and involution in mouse and human mammary cells. We determined the mRNA expression of bovine 5-HT receptor (HTR) subtypes in bovine mammary tissue (BMT) and used pharmacological agents to evaluate functional activities of 5-HT receptors. The mRNAs for five receptor isoforms (*HTR1B*, *2A*, *2B*, *4*, and *7*) were identified by conventional real-time (RT)-PCR, RT quantitative PCR, and *in situ* hybridization in BMT. In addition to luminal mammary epithelial cell expression, *HTR4* was expressed in myoepithelium, and *HTR1B*, *2A*, and *2B* were expressed in small mammary blood vessels. Serotonin suppressed milk protein mRNA expression (α-lactalbumin and β-casein mRNA) in lactogen-treated primary bovine mammary epithelial cell (BMEC) cultures. To probe the functional activities of individual receptors, caspase-3 activity and expression of α-lactalbumin and β-casein were measured. Both SB22489 (1B antagonist) and ritanserin (2A antagonist) increased caspase-3 activity. Expression of α-lactalbumin and β-casein mRNA levels in BMEC were stimulated by low concentrations of SB224289, ritanserin, or pimozide. These results demonstrate that there are multiple 5-HT receptor isoforms in the bovine mammary gland, and point to profound differences between serotonergic systems of the bovine mammary gland and the human and mouse mammary glands. Whereas human and mouse mammary epithelial cells express predominately the protein for the 5-$HT_7$ receptor, cow mammary epithelium expresses multiple receptors that have overlapping, but not identical, functional activities.

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## Introduction

Milk secretion is regulated by both systemic and local feedback mechanisms. Suckling-induced prolactin (PRL) surges comprise the major positive feedback that drives elevated milk secretion in response to increased nursing demand in most species (Wilde *et al.* 1995). Local negative feedback processes are initiated by poorly understood mechanisms that monitor the state of filling within each gland. These mechanisms regulate alveolar distension, milk synthesis and secretion, and epithelial cell mass (Peaker 1995). Acting in concert, systemic and local hormonal factors adjust mammary gland physiology not only to meet the demands of the offspring, but also to compensate for circumstances such as local mastitis, nutrient supply, and metabolic demand.

Even in the face of continued endocrine stimulation, mammary glands that are unsuckled will halt milk synthesis and undergo partial involution, resulting in loss of epithelial cell mass (Peaker 1995). In many species, including rodents and humans, involution is characterized by massive apoptosis. In other species, including dairy cattle, the glands become quiescent during involution, but do not undergo apoptosis and remodeling on a large scale (Capuco & Akers 1999). Consequences of milk stasis have been known for many years, but mechanisms responsible for stasis-induced involution have remained obscure. One line of research that explored this issue proposed the presence of a ‘feedback inhibitor of lactation’ compound in milk whose identity and physiological activity have not been confirmed. This factor was proposed to decrease milk yield, milk protein synthesis, and milk protein mRNA expression, both *in vivo* and *in vitro* and in a variety of species (Wilde *et al.* 1988).

Serotonin (5-HT) has been proposed to be an autocrine/paracrine regulator of lactation in the mouse, human, and more recently in the bovine, and the enzymatic machinery necessary for 5-HT biosynthesis has been detected in the mammary epithelium (Matsuda *et al.* 2004, Stull *et al.* 2007, Hernandez 2008, Hernandez *et al.* 2008, Pai & Horseman 2008). Expression of tryptophan hydroxylase 1 (TPH1) in mammary epithelial cells, the rate-limiting enzyme in 5-HT

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biosynthesis, was induced by PRL in mouse mammosphere cultures, and by milk stasis in nursing dams (Matsuda *et al.* 2004). Serotonin content was measured in the mammary gland of virgin, lactating, and 5-hydroxtryptophan-treated mice. Serotonin content of the mammary gland was increased in lactating mice compared with virgin and, when treated with 5-hydroxytryptophan, 5-HT levels in the mammary gland were further increased (Stull *et al.* 2007). In cultures of primary mouse mammary epithelial cells, expression of β-casein mRNA was attenuated by increasing concentrations of 5-HT. Furthermore, when methysergide (METH), a non-selective 5-HT antagonist, was added to cultures, expression of β-casein mRNA was increased (Matsuda *et al.* 2004). Non-transformed human mammary epithelial cells (MCF10A) were demonstrated to express mRNA for the human 5-HT$_7$ receptor (*HTR7*) on basolateral membranes, and 5-HT reuptake transporter (SERT) on the apical membrane (Stull *et al.* 2007). Additionally, it was determined that 5-HT was involved in the regulation of tight junction (TJ) status in the MCF10A cells. Addition of METH or metergoline, both broad-spectrum 5-HT receptor antagonists, resulted in increases in transepithelial resistance (Stull *et al.* 2007). The TJ scaffolding proteins, ZO-1 and ZO-2, were also decreased in 5-HT-treated MCF10A cells.

Studies in three-dimensional collagen cultures of bovine mammary epithelial cell (BMEC) have shown TPH1 to be expressed in a PRL-dependent manner (Stiening 2005, Hernandez *et al.* 2008). Furthermore, BMEC treated with 5-HT had decreased milk protein gene expression, and treatment with METH or parachlorophenylalanine (TPH1 enzyme inhibitor) increased milk protein gene expression (Hernandez *et al.* 2008). To date, no information is present on the identity of 5-HT receptors in bovine mammary tissue (BMT). Therefore, the objectives of these studies were to determine 1) which 5-HT receptor(s) are present in the bovine mammary gland and BMEC, 2) which selective antagonists for 5-HT receptors affect β-casein and α-lactalbumin mRNA expression in BMEC, and 3) determine whether 5-HT and selective 5-HT receptor antagonists affect caspase-3 activity in cultures of BMEC. The results of these studies point to profound functional activities of 5-HT in the bovine mammary epithelium and substantial complexity in the bovine 5-HT system, which has not yet been reported in the human or mouse 5-HT systems.

## Materials and Methods

### *Expression of HTR subtypes in bovine brain, mammary tissue, and mammary epithelial cells*

The characterization of expression for serotonergic components was initially performed on a pool of isolated bovine mammary cells representing four multiparous, non-lactating, pregnant cows. The expression patterns for 5-HT receptors in the pooled sample were confirmed in assays of four pBMEC isolates from individual cows. Additionally, expression patterns for 5-HT receptors were determined on a bovine mammary epithelial cell line (BME–UV). The morphological distribution of receptors was documented using bovine mammary and hypothalamic tissues from an individual lactating cow.

Mammary epithelial cells were collected from four multiparous, non-lactating (∼30 days dry), pregnant Holstein cows. Holstein hypothalamic (BB) and BMT were collected at slaughter from a multiparous lactating cow in the first trimester of pregnancy, and fixed at 4 °C in 4% paraformaldehyde (PFA) for 14–24 h. The tissues were submerged in 30% sucrose at 4 °C overnight, then submerged in a 1:1 v:v 30% sucrose:OCT (Tissue Tek, Sakura Finetek, Torrance, CA, USA) mixture for 24 h at 4 °C. Tissues were embedded with OCT (Tissue Tek) and frozen at −80 °C. Eight micron tissue sections were then cut with a cryostat (Microm HM 520).

Other portions of BB and BMT were used for RNA isolation and snap frozen in liquid nitrogen. Tissue was stored at −80 °C until RNA extraction.

Total RNA was isolated from BB, BMT, BMEC, and BME–UV samples using TRIzol reagent (Invitrogen) in triplicate. For primary epithelial cell culture experiments, two wells of a 24-well culture plate served as one sample and there were four samples for each treatment. In a given experiment, each treatment was represented in each culture plate, resulting in four culture plates per study. Samples were stored at −80 °C until extraction. Extraction was conducted as described previously (Hernandez *et al.* 2008).

Quantitative real-time (RT)-PCR analysis was conducted using the iCycler IQ RT-PCR Detection System (Bio-Rad). Hypoxanthine phosphoribosyltransferase I (*HPRT1*) was utilized as the internal control gene following standard curve analysis across all treatment group samples. Several other housekeeping genes were evaluated (ribosomal protein S18, glyceraldehyde 3 phosphate, and β-actin) and *HPRT1* did not alter its expression based on treatment. Resulting gene expression data were calculated and analyzed based on the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen 2001). Amplification efficiencies of primers were evaluated prior to conducting experiment to determine equality of internal control compared with primers. All primers utilized met criteria for analysis by the $2^{-\Delta\Delta C_t}$ method, with efficiencies between 95 and 105% (Livak & Schmittgen 2001). Amplicons were sequenced from RT-PCR products prior to the use of primers for quantitative PCR (qPCR). Primer sequences are shown in Table 1.

BMT was counterstained after *in situ* hybridization for smooth muscle α-actin (SMA) to identify myoepithelial cells and for Griffonia simplicifolia lectin I isolectin B4 (GS-I) to identify vasculature in BMT. Primary biotinylated antibody was obtained (#B-1205; Vector Laboratories, Burlingame, CA, USA) for biotinylated GS-I. Primary antibody for SMA (#MS-113-P0; Thermo Scientific, Fremont, CA, USA) was visualized with a biotinylated goat anti-rabbit secondary antibody (#170-6401; Bio-Rad Laboratories) for immunohistochemical detection in BB and BMT following *in situ*

**Table 1** Primers utilized for RT-PCR and qPCR analyses. All 5-HT receptors were run at an annealing temperature of 64 °C. Primers for 5-HT receptors 1A–4 were obtained from Reist *et al.* (2003) and specific to the bovine genome. The 5-$HT_7$ was designed using primer 3 (Rozen & Skaletsky 2000) and the sequence was obtained from GenBank (NIH, Bethesda, MD, USA), accession number XM_ 580794. HPRT1 was used as the housekeeping gene and was also run at an annealing temperature of 64 °C. β-Casein was run at a 64 °C annealing temperature and α-lactalbumin was run at a 62 °C annealing temperature

| **Primer** | **Forward primer** (5′ to 3′) | **Reverse primer** (5′ to 3′) |
|---|---|---|
| *HTR1A* | TCAGCTACCAAGTGATCACCTCT | GTCCACTTGTTGAGCACCTG |
| *HTR1B* | TGCTCCTCATCGCCCTCTATG | CTAGCGGCCATGAGTTTCTTCTT |
| *HTR1D* | CCTCCAACAGATCCCTGAATG | CAGAGCAATGACACAGAGATGCA |
| *HTR1F* | TGTGAGAGAGAGCTGGATTATGG | TAGTTCCTTGGTGCCTCCAGAA |
| *HTR2A* | AGCTGCAGAATGCCACCAACTAT | GGTATTGGCATGGATATACCTAC |
| *HTR2B* | AAACAAGCCACCTCAACGCCT | TCCCGAAATGTCTTATTGAAGAG |
| *HTR2C* | TTCTTAATGTCCCTAGCCATTGC | GCAATCTTCATGATGGCCTTAGT |
| *HTR4* | ATGGACAAACTTGATGCTAATGTGA | TCACCAGCACCGAAACCAGCA |
| *HTR5a* | ACAACGGGGACATCTAGGG | TTGGGACATGGTAAGTACTAGGG |
| *HTR7* | GTTTTATATCCCCATGTCCGTCA | TTTGCACACTCCTCTACCTCCT |
| β-Casein | GCTATGGCTCCTAAGCACAAAGA | GGAAACATGACAGTTGGAGGAAG |
| α-Lactalbumin | CTCTGCTCCTGGTAGGCATC | ACAGACCCATTCAGGCAAAC |
| *HPRT1* | GAGAAGTCCGAGTTGAGTTTGGAA | GGCTCGTAGTGCAAATGAAGAGT |

hybridization for 5-HT receptors. The Vectastain Elite ABC Kit (Vector Laboratories) was used for staining following the manufacturer's instructions, and the following primary antibody dilutions were utilized: 3 μg/ml GS-I; 1:800 SMA. Proteins were visualized using DAB peroxidase substrate per manufacturer's instructions (Vector Laboratories).

### In situ *hybridization for HTR in BMT*

*HTR1B*, *2A*, *2B*, *4*, and 7 cDNA clones were generated by PCR amplification from total RNA extracted from BB. Primers for specific genes of interest were designed against bovine sequences for *HTR1B*, *2A*, *2B*, and *4* receptors as reported in National Center for Biotechnology Information (NCBI; Bethesda, MA, USA). The *HTR7* receptor was designed using human sequences for the receptor and compared with bovine genome (NCBI). The *HTR7* receptor primer was generated from primer 3 (Rozen & Skaletsky 2000). The following primer sequences were used for probe generation: *HTR1B* were 5′-ATGGAGAAGACCCACACCAG and GTGATTGCCACTGTGTACCG-3′; *HTR2A* were 5′-TTCTCCCTGACTCCTCAAAACTG and GGCATTCTGCAGCTTTTTCTCTA-3′; *HTR2B* were 5′-CGATTCTGCCACAACAAGAA and TTCCCTGTTCCTCACCAGTC-3′; *HTR4* were 5′-CTCTGGATGTCCTGCTCACA and CATGCGATGAGTGCTATGCT-3′; and *HTR7* were 5′-CTTTGGCCATTTCTTCTGTAACG and TGGAGATGTTTTTCCTTTCGTGT-3′. Amplified DNA products for 5-HT receptors were inserted into the TOPO TA cloning expression vector pCRII (Invitrogen) and chemically transformed into Mach1-T1 *Escherichia coli* (Limesand *et al*. 2005). Plasmids positive for PCR inserts were transformed and plasmid DNA was isolated (Qiagen) and sequenced. Chromas and BLAST (NIH) were used to analyze DNA sequences generated from PCR and confirm sequence identity.

RNA probes were synthesized using plasmid DNA that was linearized using endonuclease restriction enzymes and extracted. Digoxigenin (DIG)-labeled RNA probes (Roche Diagnostics) were generated with SP6 or T7 RNA polymerase (Promega), precipitated with 70% ETOH, and resuspended. Probe absorbance was measured with a NanoDrop Spectrophotometer ND-1000 to determine concentration and integrity.

*In situ* hybridization was conducted on frozen sections fixed in 4% PFA for 10 min, washed in 1×PBS, digested with 10 μg/ml proteinase K for 10 min, and refixed in 4% PFA for 5 min. Tissues were washed in PBS and then acetylated (102·2 mM triethanolamine, 0·01 mM 6 M HCl, and 26·9 mM acetic anhydride) for 10 min at room temperature, washed in PBS, and then blocked with 55 °C pre-hybridization buffer (50% formamide, 5×sodium chloride/sodium citrate (SSC), pH 4·5, 50 μg/ml yeast tRNA, 1% SDS, and 50 μg/ml heparin) for 2 h at 55 °C in a humidified chamber. The DIG-labeled RNA for sense (negative control) and anti-sense strands for each receptor was then separately added to hybridization buffer (*HTR1B*: anti-sense 66·80 ng/μl, sense 92·01 ng/μl; *HTR2A*: anti-sense 19·5 ng/μl, sense 103·1 ng/μl; *HTR2B*: anti-sense 84·59 ng/μl, sense 150·40 ng/μl; *HTR4*: anti-sense 56·15 ng/μl, sense 78·40 ng/μl; *HTR7*: anti-sense 68·30 ng/μl, sense 63·09 ng/μl), heat denatured at 80 °C for 5 min, cooled, then hybridization buffer with DIG-RNA for sense and anti-sense strands for each receptor was added to separate slides on tissue sections and incubated overnight at 70 °C in a humidified chamber. Sections were washed in 70 °C 5×SSC, pH 7·0, for 30 min at room temperature, incubated in 0·2×SSC, pH 7·0, for 3 h at 75 °C, then washed at room temperature in 0·2×SSC for 5 min. Tissues were subsequently washed in 1×malic acid buffer (MAB), pH 8·0, then incubated in blocking buffer consisting of 2% blocking reagent in MAB (Roche Diagnostics), 10% heat inactivated FBS (Gibco), 0·1% Tween-20 for 1 h at room temperature. Anti-DIG-AP Fab

Fragments antibody (Roche Diagnostics) was diluted 1:1000 in blocking buffer and incubated at 4 °C overnight in the dark. Following incubation, BB and BMT sections were washed in MAB with 0·1% Tween-20, then in DI water with 0·1% Tween-20 and then developed with BM Purple AP Substrate (Roche Diagnostics) containing 0·1% Tween-20 for 3–36 h until maximum intensity was achieved.

### *Effects of serotonergic drugs on primary BMECs in collagen gel cultures*

Tissue dissociation, BMEC isolation, and preparation of type 1 collagen were performed according to (McGrath 1987, Hernandez *et al.* 2008, Stiening *et al.* 2008). Epithelial cells in the form of organoids clumps composed of ductal and alveolar cells were isolated from four multiparous, non-lactating (∼30 days dry), pregnant Holstein cows. Organoids were thawed, resuspended in DMEM/F-12, mixed with neutralized collagen at 4–6×$10^5$ organoids/ml of collagen mixture, and cultured in 24-well plates as described previously (Hernandez *et al.* 2008, Stiening *et al.* 2008). The average DNA content of one vial of BMEC is 486·7±84·91 μg/ml (mean±S.D). This provided sufficient cells for 72 wells or ∼6·8 μg DNA/well.

Mammary cell organoids were allowed to grow for 8 days in serum-free media (100 ng/ml insulin-like growth factor-I (IGFI) and 25 ng/ml epidermal growth factor (EGF)), and then treated with a lactogenic hormone complex (100 ng/ml IGFI, 100 ng/ml PRL, and 10 ng/ml hydrocortisone, without EGF) plus gel release from the plastic by rimming of the gels from the plastic, simultaneously, for 48 h in combination with and without 5-HT receptor antagonists, as previously described (Hernandez *et al.* 2008). The lactogenic hormone complex in serum-free media served as the control, and 5-HT antagonists in combination with the lactogenic complex served as treatments, with the entire experiment being replicated an additional time. At termination of lactogenic treatments, gels were dissolved in 1 ml TRIzol for 5 min (Invitrogen) and stored at −80 °C until RNA extraction. Two gels were utilized per replicate and four replicates per treatment were completed. For caspase activity assays, collagen gels were digested with 0·1% collagenase I (collagenase type 1, Worthington Biochemical Corp., Lakewood, NJ, USA) in Hank's balanced salt solution with 4% BSA for 20 min at 37 °C (Rocha *et al.* 1985). This mixture was centrifuged for 4 min at 100 ***g*** and supernatant removed. Cells were washed with M199, centrifuged for 4 min at 100 ***g***, and supernatant removed. Cell pellets were stored at −80 °C until caspase-3 activity assay was conducted. Two wells of a 24-well plate served as an $N=1$, and an $N=4$ was completed (eight total gels) for each treatment group for the two experimental replicates.

The effects of 5-HT (Sigma; 200 μM) and selective 5-HT receptor antagonists/reverse agonists for the 1B (SB224289; Sigma), 2A (ritanserin; Sigma), 2B (SB204741; Sigma), four (SB204070; Sigma), and seven (pimozide; Sigma) receptors were investigated during the lactogenic period of culture (48 h) in combination with lactogenic hormones. A 200 μM concentration of 5-HT was utilized as this was determined to be an effective concentration in other mammary epithelial cell models (Matsuda *et al.* 2004, Hernandez *et al.* 2008, Pai & Horseman 2008). Concentrations of 5-HT receptor antagonists ranged from 0·5 to 10 μM, except for SB204070, which ranged from 0·0001 to 1·0 μM. Cultures treated with lactogenic media only, plus gel release served as the control. Effects of specific 5-HT receptor antagonists/reverse agonists on expression of mRNA for milk protein genes α-lactalbumin and β-casein during lactogenesis were also investigated.

For caspase-3 activity measurements, the following concentrations of 5-HT and 5-HT receptor antagonists were utilized during lactogenic treatment: 200 μM 5-HT (Sigma), 0·1 μM pimozide, 1·0 μM ritanserin, 0·0001 μM SB204070, 0·1 μM SB204741, and 1·0 μM SB224289. Cultures treated with lactogenic media only, plus gel release served as the control. At termination of lactogenic treatments, gels were harvested and treated for protein isolation for caspase-3 activity and stored at −80 °C.

Activation of caspase-3 was quantified using BioMol QuantiZyme Colormetric Assay kit (BioMol, Plymouth Meeting, PA, USA), measuring the rate of Ac-DEVD-pNA cleavage. Caspase-3 is a committed step for caspase activity in cells that undergo apoptosis. Methods utilized were based upon (Limesand *et al.* 2003).

### *Statistical analysis and 5-HT nomenclature*

Statistical analysis was conducted using a one-way ANOVA on qPCR data using gene expression relative to the control (lactogenic media+gel release) in a respective sample, with the PROC MIXED procedure of SAS (SAS, 9.3, SAS Institute, Cary, NC, USA). Graphical representation of data is represented by expression of treatments relative to the control ($2^{-\Delta\Delta C_t}$). The $\Delta\Delta C_t$ was calculated as $\Delta C_t$ of a respective treatment minus $\Delta C_t$ of the control. A one-way ANOVA was conducted on results from caspase-3 activity assay, with the PROC MIXED procedure of SAS (SAS, 9.3, SAS Institute).

Nomenclature utilized for the mRNA 5-HT receptors (*HTR* humans/*HTR* non-human species) was derived from the NCBI. Nomenclature utilized for the protein form of the 5-HT receptors (5-$\text{HT}_\text{R}$) was derived from the International Union of Basic and Clinical Pharmacology (www.iuphar.org, IUPHAR, Kansas City, KS, USA).

## Results

### *Expression of 5-HT receptors in the bovine mammary gland*

Serotonin exerts its cellular actions through multiple receptor subtypes belonging to seven different families. Determination of 5-HT receptor subtypes present in BMEC and BMT was a necessary first step for establishing the basis of 5-HT actions in

BMT development and lactation after having determined that BMEC expresses TPH-1, the rate-limiting enzyme for 5-HT biosynthesis and the serotonin re-uptake transporter, necessary for 5-HT recycling into the cell (Hernandez 2008, Hernandez *et al.* 2008). To validate detection of receptor subtypes by RT-PCR, BB served as a control because all 5-HT receptors are present in brain tissue (Kroeze *et al.* 2002). Receptor types that were validated by amplifying BB cDNA included *HTR1A*, *1B*, *1D*, *1F*, *2A*, *2B*, *2C*, *4*, *5A*, and 7 (Fig. 1 and data not shown). These primers were used for detecting mRNA expression in BMT. Other receptors that were not included in these assays (e.g. types 3, 6, and a variety of subtypes) may be expressed, but further validation studies would be necessary to develop adequate assays.

Expression of genes encoding the proteins for $5\text{-HT}_{1B,\ 2A,\ 2B,\ 4,\ 7}$ receptors was detected in BMT and BMEC (Fig. 1). The mRNAs for *HTR1A*, *1D*, *1F*, *2C*, and *5A* receptors were not detected in BMT or BMEC (data not shown). Additionally, expression of genes encoding the proteins for $5\text{-HT}_{1B,\ 2A,\ 2B,\ 4,\ 7}$ receptors was detected in a BME–UV (data not shown). Additionally, mRNA for *HTR1D* was detected in BME–UV cells (data not shown).

Relative expression levels for each receptor of the five subtypes that were expressed in BMT were determined using qPCR. Levels of each receptor mRNA were quantified as the ratio of BMT expression to the level in BB extracts. The expression ratio of receptor mRNA relative to BB for each type in BMT, highest to lowest, was as follows: *HTR4*, 389; *HTR2B*, 89; *HTR1B*, 19·35; *HTR7*, 18; and *HTR2A*, 3·8. In BMEC, the expression ratio relative to BB (highest to lowest) was: *HTR4*, 230; *HTR2B*, 44; *HTR7*, 37; *HTR1B*, 30; and *HTR2A*, 1·6. The rank order of expression ratios was similar for most of the receptors in both BMT and BMEC, with the exception of *HTR7*, which was enriched in BMEC. The relative enrichment of $5\text{-HT}_7$ in epithelial cells is consistent with expression of this receptor in the mammary epithelium in mice and humans (Stull *et al.* 2007, Pai & Horseman 2008).

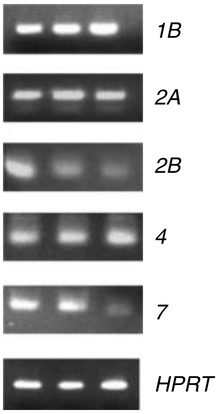

**Figure 1** 5-HT receptor expression in bovine hypothalamic tissue, lactating mammary tissue, and primary bovine mammary epithelial cells. The mRNA expression of the HTR was detected by RT-PCR and electrophoresed through a 2% agarose gel and stained with EtBR (primers listed in Table 1). Lane (A) Holstein hypothalamic tissue, Lane (B) lactating Holstein mammary gland tissue from a first trimester pregnant animal, and Lane (C) primary bovine mammary epithelial cells isolated from three multiparous, non-pregnant (~30 days dry), Holstein cows treated for 7 days with serum-free proliferation media (IGF-I, EGF and INS).

Having determined that genes encoding proteins of several 5-HT receptor subtypes were expressed in BMT, it was necessary to determine what cell types expressed each. Therefore, *in situ* hybridization assays for *HTR1B*, *2A*, *2B*, *4* and 7 were conducted. BB was used as a positive control and sense probes for receptors were used as negative controls (Fig. 2). Sections for each receptor probe were counterstained with GS-I or SMA to identify vascular endothelial and myoepithelial cells respectively (Fig. 2).

Expression of *HTR1B*, *2A*, *2B*, *4*, and 7 was each localized in BMT (Fig. 2, row A, columns 1–5). Counterstaining with GS-I (vascular endothelium) indicated that *HTR1B*, *2A*, and *2B* were present in the endothelium in addition to epithelium (Fig. 2). Myoepithelium expressed *HTR4* (Fig. 2).

### *Agonist effects on milk protein mRNA expression and apoptosis*

Serotonin has been demonstrated to decrease milk protein mRNA expression in primary mouse mammary epithelial cells and to induce apoptosis human and mouse mammary epithelial cells (Matsuda *et al.* 2004, Stull *et al.* 2007). Therefore, it was pertinent to determine whether 5-HT treatment of BMEC would also result in decreased milk protein mRNA expression and increased apoptosis.

Treatment of lactogenic collagen gel cultures of BMEC (lactogenic hormones, plus gel release) with exogenous 5-HT depressed β-casein mRNA expression by >90% compared with BMEC treated with lactogenic conditions only ($P<0{\cdot}0001$; data not shown). Similarly, 5-HT suppressed α-lactalbumin mRNA by >80% in comparison with lactogenic control ($P<0{\cdot}0001$; data not shown).

When lactogenic cultures of BMEC were treated with exogenous 5-HT, there was no significant increase in apoptosis as measured by caspase-3 activity ($P=0{\cdot}10$).

### *Effects of 5-HT receptor antagonists on milk protein mRNA expression and apoptosis in primary BMECs*

The effects of selective 5-HT antagonists/inverse agonists were tested in collagen gel cultures of BMEC under lactogenic conditions (lactogenic hormone stimulation plus gel release). For each receptor, an antagonist was chosen based on its relative selectivity for the target receptor compared with other receptors that were identified in BMEC. Antagonists utilized were as selective for a specific 5-HT receptor of interest as possible. Concentration-response curves for each antagonist were performed using a range of concentrations previously documented to encompass established $IC_{50}$ for the target receptor. The antagonists and their respective receptors included SB224289

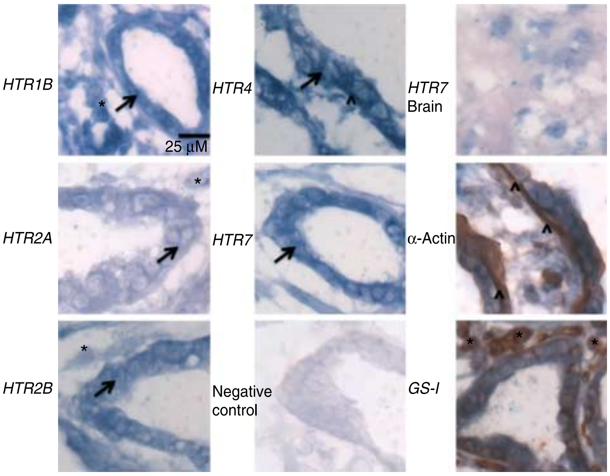

**Figure 2** Morphological distribution of HTR in lactating bovine mammary tissue. *In situ* hybridizations of DIG-labeled anti-sense cRNA probes for *HTR1B*, *HTR2A*, *HTR2B*, *HTR4*, and *HTR7*, as well as representative anti-sense cRNA DIG-labeled probe in the mammary gland. Anti-sense cRNA-labeled probe for HTR7 in the bovine hypothalamus is also provided. A representative section that has been stained for HTR7 and has been counterstained for smooth muscle α-actin and GS-I is shown. Black arrows (→) indicate epithelial cells. Arrowheads (^) represent myoepithelial cells, and asteriks (*) correspond to vascular endothelium.

($5\text{-HT}_{1B}$), SB204070 ($5\text{-HT}_4$), ritanserin ($5\text{-HT}_{2A}$), SB204741 ($5\text{-HT}_{2B}$), and pimozide ($5\text{-HT}_7$).

In the case of $5\text{-HT}_{1B}$, SB224289, at 0·5, 1·0, and 2·0 µM, up-regulated α-lactalbumin mRNA expression 10, 11, and 22-fold respectively ($P<0{\cdot}05$; Fig. 3A) relative to lactogenic control. Exogenous 1·0 and 2·0 µM ritanserin ($5\text{-HT}_{2A}$ receptor antagonist) increased α-lactalbumin mRNA levels relative to lactogenic control 17 and 11-fold respectively ($P<0{\cdot}001$ respectively; Fig. 3B). Pimozide ($5\text{-HT}_7$ receptor antagonist) at 0·2 µM increased α-lactalbumin mRNA 16-fold compared with lactogenic control ($P<0{\cdot}001$; Fig. 3C). We failed to detect changes in α-lactalbumin mRNA expression when BMEC was treated with exogenous antagonists to $5\text{-HT}_{2B}$ and $5\text{-HT}_4$ receptor subtypes (data not shown).

β-Casein mRNA expression was up-regulated relative to the lactogenic control when treated with 1·0 and 2·0 µM SB224289 ($P<0{\cdot}05$; Fig. 4A). Additionally, antagonism of the $5\text{-HT}_{2A,7}$ receptors with exogenous ritanserin (2·0 µM) and pimozide (0·1 and 0·2 µM) significantly increased β-casein mRNA expression (Fig. 4B and C). We were unable to detect significant effects on β-casein mRNA expression when BMEC was treated with antagonists to $5\text{-HT}_{2B,4}$ receptor subtypes (data not shown).

Treatment of BMEC with 1·0 µM $5\text{-HT}_{1B}$ and $5\text{-HT}_{2A}$ receptor antagonists increased caspase-3 activity ($P<0{\cdot}05$ and $P<0{\cdot}001$ respectively; Fig. 5). All other 5-HT receptor antagonists did not affect caspase-3 activity, a marker of apoptosis ($P>0{\cdot}05$; Fig. 5).

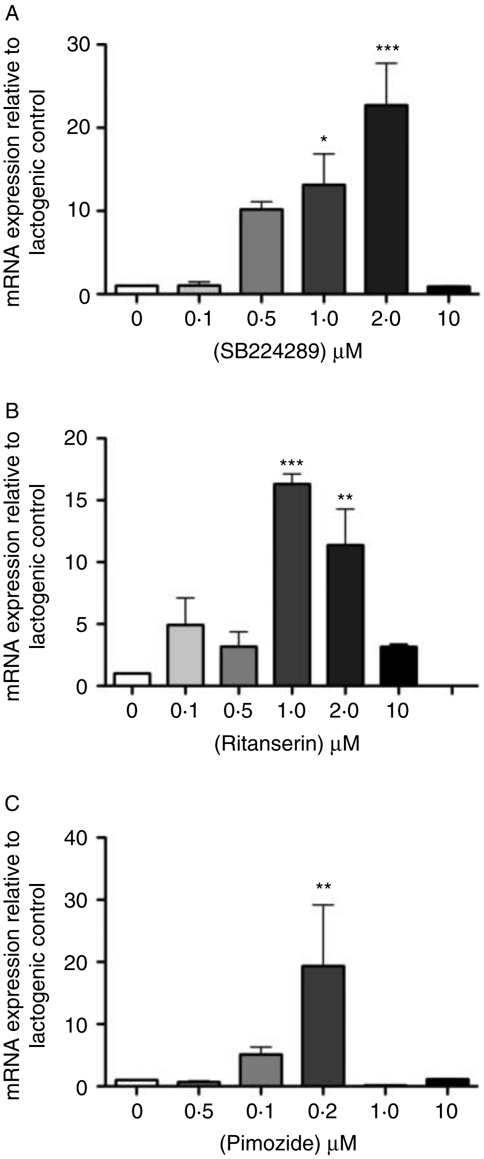

**Figure 3** Effects of selective 5-HT receptor antagonists on α-lactalbumin mRNA expression in primary bovine mammary epithelial cells. Mean with S.E.M. of (A) α-lactalbumin mRNA expression in lactogenic BMEC cultures treated with SB224289, a 5-HT-1B receptor antagonist; (B) α-lactalbumin mRNA expression in lactogenic BMEC cultures treated with ritanserin, a 5-HT-2A receptor antagonist; and (C) α-lactalbumin mRNA expression in lactogenic BMEC cultures treated with pimozide, a 5-HT-7 receptor antagonist. Columns with * indicate statistical significance of $*P<0{\cdot}05$, $**P<0{\cdot}001$, $***P<0{\cdot}0001$ compared with lactogenic control.

## Discussion

Recently, the $5\text{-HT}_7$ receptor has been discovered in human and mouse mammary epithelium (Stull *et al.* 2007). Furthermore, it was demonstrated that the mammary gland epithelial cells contain the enzymatic machinery to produce

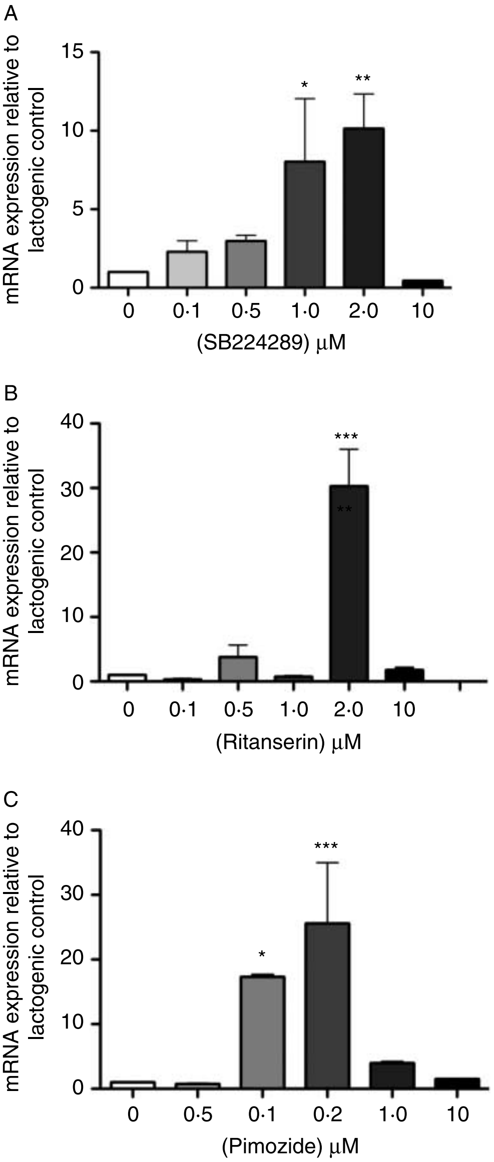

**Figure 4** Effects of selective 5-HT receptor antagonists on β-casein mRNA expression in primary bovine mammary epithelial cells. Mean with S.E.M. of (A) β-casein mRNA expression in lactogenic BMEC cultures treated with SB224289, a 5-HT-1B antagonist; (B) β-casein mRNA expression in lactogenic BMEC cultures treated with ritanserin, a 5-HT-2A receptor antagonist; and (C) β-casein mRNA expression in lactogenic BMEC cultures treated with pimozide, a 5-HT-7 receptor antagonist. Columns with *Indicate statistical significance of *$P<0{\cdot}05$, **$P<0{\cdot}001$, ***$P<0{\cdot}0001$ compared with lactogenic control.

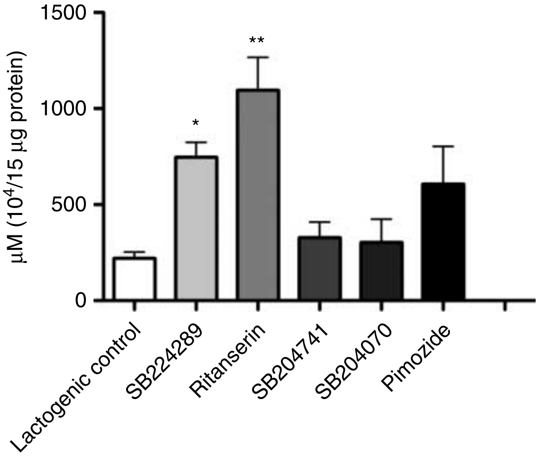

**Figure 5** Caspase-3 activity in primary bovine mammary epithelial cells treated with 5-HT receptor antagonists. Mean with S.E.M. of caspase-3 activity in lactogenic cultures of BMEC treated with 1·0 µM SB224289, 1·0 µM ritanserin, 0·1 µM SB204741, 0·0001 µM SB204070, and 0·1 µM pimozide. Columns with * indicate statistical significance of *$P<0{\cdot}05$; ***$P<0{\cdot}0001$.

5-HT (TPH1, aromatic amino acid decarboxylase, and SERT), and that more 5-HT is detectable in the mammary gland during lactation (Matsuda *et al.* 2004, Stull *et al.* 2007, Hernandez 2008, Hernandez *et al.* 2008). Additionally, it was demonstrated that 5-HT disrupts mammary epithelial TJ, and this occurs through action of 5-HT$_7$ (Pai & Horseman 2008). In cattle, 5-HT has been demonstrated to reduce milk production in late lactation and to negatively impact milk protein gene expression in BMEC cultures (Hernandez *et al.* 2008).

We tested BB and BMT isolated from a single pregnant, lactating Holstein cow, and BMEC isolated from four non-lactating, pregnant Holstein cows, cultured in collagen gels, as well as a BME-UV, for mRNA for the *HTR1A*, *1B*, *1D*, *1F*, *2A*, *2B*, *2C*, *4*, *5A*, and *7*. We detected mRNA of all 5-HT receptors in BB. mRNA for the *HTR1B*, *2A*, *2B*, *4*, and *7* was detected in both BMT and BMEC by RT-PCR (Fig. 1). All five receptors were also localized to the mammary epithelium by *in situ* hybridization (Fig. 2), suggesting a very complex local regulation of the bovine mammary gland by 5-HT. We have previously demonstrated that *in vivo* intramammary infusions of 5-HT resulted in a 10% decrease in milk yield in late-lactation Holstein cows, and METH resulted in a 10% increase in milk yield (Hernandez *et al.* 2008).

Herein, we report that 5-HT suppressed transcription of milk protein genes ($P<0{\cdot}0001$). Serotonin has also been reported to increase apoptosis in mouse mammary explant cultures (Matsuda *et al.* 2004). In three-dimensional lactogenic collagen cultures of BMEC treated with 200 µM 5-HT, we saw no significant increase in caspase-3 activity relative to lactogenic controls ($P=0{\cdot}10$). Although contrary to the extensive apoptosis seen in mice upon treatment with 5-HT, there may be a solid biological basis for this difference. Dairy cows do not undergo extensive remodeling of BMT to a virgin-like state during their involution/dry period (Akers 2002). No evidence of a net loss of mammary epithelial cells is apparent in lactating versus non-lactating cows. Furthermore, dairy cows undergo more extensive cell turnover during lactation than mice or humans (Capuco *et al.* 1997, 2006).

Therefore, the lack of extensive apoptosis in BMEC treated with 5-HT is not entirely surprising.

We determined that *HTR1B* mRNA was enriched in BMEC and in BMT, suggesting that it is predominately found in BMEC, although it appears to be also expressed in the vasculature of the mammary gland. We demonstrated that SB224289 (1B antagonist) up-regulated α-lactalbumin and β-casein mRNA expression in BMEC exposed to a lactogenic treatment (Figs 3A and 4A). Increasing cyclic AMP enhances secretion in mammary epithelial cells, and potentially 5-HT$_{1B}$ could be blocking this pathway because it is negatively coupled to cAMP production (Boisgard *et al.* 2001). In these studies, we demonstrated that treatment of BMEC with SB224289 increased apoptosis compared with lactogenic controls (Fig. 5). In the bovine, a considerable amount of cell renewal occurs, such that by the end of lactation, the majority of cells present has been produced during the lactation cycle (Capuco *et al.* 2001, 2003) Apoptosis is crucial to turnover of cells in BMT during lactation (Capuco *et al.* 2003, Hadsell *et al.* 2007). Additionally, it has been suggested that increasing cell turnover in BMT could aid in increasing secretory capacity in individual BMEC or could increase replacement of less functional cells (Capuco *et al.* 2006).

The 5-HT$_{2A}$ has low affinity for 5-HT and has been shown to activate phospholipases (A$_2$, C, and D), and therefore increase intracellular Ca$^{2+}$ levels, activate Janus kinase/signal transducers and activators of transcription (Jak/Stat) pathway, and increase glucose uptake by skeletal muscle by increasing glucose transporters (GLUT) 1, 3, and 4 in the plasma membrane (Hadjuch *et al.* 1999, Raymond *et al.* 2001, Banes *et al.* 2005). Glucose uptake by BMT is a required step in producing lactose, the primary carbohydrate and major osmolyte in milk. The major GLUT in the mammary gland is GLUT1 (Akers 2002). PRL stimulates the Jak/Stat pathway (Jak2/Stat5) in the mammary gland, which is responsible for the induction of milk protein expression in BMEC as well as other mammary epithelial cells due to the presence of Stat5-binding sites on the epithelial cells, and this could be a potential regulatory pathway by which 5-HT acts through 5-HT$_{2A}$ (Horseman 1999). Our studies indicate that the mRNA for the *HTR2A* is present in BMT and potentially in cells of the vascular system of BMT (Fig. 2). Expression of mRNA for this receptor was 3·77-fold in BMT and 1·64 in BMEC relative to BB. We observed that treatment of BMEC with ritanserin increased α-lactalbumin and β-casein mRNA compared with lactogenic controls (Figs 3B and 4B). The 5-HT$_{2A}$ is involved in contraction of vascular smooth muscle cells through ERK/MAPK pathway (Watts 1998). This action could be involved in the regulation of blood flow to the mammary gland, which is important in supporting lactation (Collier *et al.* 1984).

We detected the presence of *HTR2B* mRNA in BMT (Fig. 2). These receptors are also involved in regulation of morphogenesis and mitogenesis, and stimulate ERK and cell cycle components (Raymond *et al.* 2001). We did not detect any effects on milk protein gene expression or milk protein levels when treating lactogenic cultures of BMEC with SB204741 (2B antagonist). Thus, this receptor may not be involved in the regulation of milk protein genes in the bovine, but other processes not measured.

We identified the *HTR4* mRNA in BMT as well as BMEC, and potentially in the myoepithelium (Fig. 2). However, no effects were observed on milk protein gene expression or apoptosis when BMEC was treated with a 5-HT4 receptor antagonist. This may also not be surprising since we did not measure the milk ejection reflex in these studies.

The 5-HT$_7$ receptor subtype is positively coupled to adenylyl cyclase through $G_s$, and has been detected in both human and mouse mammary epithelial cells on the basolateral side of the membrane (Stull *et al.* 2007). We also detected *HTR7* mRNA in BMT and BMEC (Fig. 2). Additionally, it has been demonstrated that 5-HT, through cAMP, regulates TJ status in human mammary epithelial cells in a biphasic manner, with long-term exposure to 5-HT resulting in a disruption of TJ status through a p38 MAPK pathway (Pai & Horseman 2008). We detected an increase in α-lactalbumin and β-casein mRNA expression when BMEC was treated with a 5-HT$_7$ antagonist (Figs 3C and 4C). In epithelial cells, cAMP and protein kinase A have been demonstrated to stimulate apically directed transcytosis and secretion (Muniz *et al.* 1996). Furthermore, it has been demonstrated that an increase in cAMP results in increased casein secretion in mammary epithelial cells through protein kinase A (Boisgard *et al.* 2001).

Serotonin receptor activation patterns *in vivo* are unknown, and could effect milk protein gene expression variably. In addition to tissue-autonomous mechanisms explored to date, some mechanisms of 5-HT action may involve extra-epithelial actions. For instance, 5-HT acts as a mammary artery vasoconstrictor, which severely decreases mammary blood flow, a correlate for decreased capacity for milk production (Linzell 1974, Collier *et al.* 1984).

## Conclusion

We have demonstrated the basic properties of the serotonergic signaling system in dairy cows. In this process, we have discovered profound differences between the bovine system and that of mice and humans. Whereas human and mouse mammary epithelial cells express predominantly the 5-HT-7, which signals through the $G_s$-coupled pathway, the bovine mammary epithelium expresses 5-HT-7, along with four additional isoforms of 5-HT receptors (1B, 2A, 2B, and 4). These additional receptors may signal through the $G_i$ and $G_{q/11}$ pathways, raising the likelihood that cows have evolved mechanisms that have served to alter the molecular tuning of intramammary serotonergic feedback. We have further demonstrated that 5-HT-1B, 2A, and 7 appear to be directly involved in milk protein gene expression in the bovine.

Other potential mechanisms of serotonergic regulation of mammary gland function include cell turnover, the milk ejection reflex, and mammary blood flow. Further studies will be needed to delineate the exact mechanisms of serotonergic regulation of the bovine mammary gland.

## Declaration of interest

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

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