Suppression of lactation and acceleration of involution in the bovine mammary gland by a selective serotonin reuptake inhibitor

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Abstract

Serotonin (5-HT) is a homeostatic regulator of lactation. Selective 5-HT reuptake inhibitors (SSRI) are commonly prescribed pharmaceuticals that inhibit activity of the 5-HT reuptake transporter, increasing cellular exposure to 5-HT. Use of SSRIs has been shown to alter lactation performance in humans and 5-HT has been shown to reduce milk yield in cattle. However, it has not been determined how SSRI treatments affect the bovine mammary gland. We evaluated the effects of SSRI (fluoxetine (FLX)) administration on tight junctions (TJs) and milk protein gene expression in a lactogenic culture model, using primary bovine mammary epithelial cells (pBMEC). Additionally, we evaluated the effects of intramammary infusions of FLX and 5-hydroxytryptophan on milk production and TJ status in multiparous Holstein cows at dry-off. Treatment of pBMEC cultured on

permeable membranes disrupted TJs, as measured by transepithelial resistance and immunostaining for zona occludens 1. Correspondingly, treatment of '3D', collagen-embedded lactogenic cultures of pBMEC with FLX suppressed milk protein gene expression (α -lactalbumin and β -casein) in a concentration-dependent manner. Finally, intramammary treatment of Holstein cows with FLX resulted in an accelerated rate of milk decline. Additionally, TJ permeability increased in FLX-treated animals, as measured by plasma lactose and milk Na^+ and K^+ levels. Results of these experiments imply that SSRI administration accelerates the rate of mammary gland involution through disassembly of TJs and inhibition of milk protein gene expression *in vitro* and *in vivo*, leading to reduction of milk yield.

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Introduction

Mammary gland involution is a complex and critical process that the gland undergoes at the cessation of its lactation cycle, or when demand for milk decreases. The process in laboratory rodents is associated with a high level of epithelial cell apoptosis and extensive remodeling of the gland (Stein *et al.* 2007). However, the morphological changes at involution in dairy animals (cattle, goats and sheep) are less pronounced than in rodents. The changes in humans are intermediate between rodents and dairy animals (Capuco & Akers 1999). Involution in dairy animals is characterized by a low and relatively continuous rate of epithelial cell turnover and mammary gland morphological maintenance, rather than a large-scale tissue regression process as seen in rodents (Capuco & Akers 1999). The morphological changes that occur in the mammary gland of dairy animals at involution largely reflect a change in the secretory state of the gland, rather than the tissue remodeling process (Capuco & Akers 1999). Apparent differences among species partially reflect whether involution is concurrent with pregnancy under

typical experimental conditions. Involution and mammary cell turnover is a critical process in the cycle of the mammary gland in dairy animals, which must undergo a dry period in order to avoid losses in milk production in their subsequent lactation cycle (Collier *et al.* 2004).

Intramammary infections contracted during the dry period have been associated with increased rates of infection during the subsequent lactation, and therefore large losses in milk production (Eberhart 1986). Currently, mastitis is the costliest disease in the dairy industry, resulting in losses over \$2 billion a year in the United States (Rainard 2005). Furthermore, mastitis often occurs in lactating women during periods of insufficient drainage, rapid weaning, oversupply of milk, pressure on the breast, blocked ducts and lengthy intervals between feedings (Betzold 2007). The incidence of mastitis increases significantly during periods of milk stasis both in dairy animals and in humans (Oliver & Sordillo 1988, Betzold 2007). In fact, of all enterobacterial mastitis infections, 52.6% arise in glands previously infected during the dry period in dairy animals (Bradley & Green 2000). Furthermore, dairy animals producing more milk at the time they end the dry

period, as well as individual quarters/glands that are producing more milk, are more susceptible to intramammary infections (Oliver & Smith 1982). Mastitis ranges from 2 to 33% of lactating women who are exposed to extended intervals of milk stasis (Betzold 2007).

Acceleration of involution would be important for protection against mastitis in humans during weaning and in dairy animals during the dry period. Currently, dairy animals are subjected to frequent milking intervals followed by abrupt cessation of milking in combination with antibiotic treatment, and in some cases feed restriction, to reduce milk secretion and initiate the involution period (Dingwell *et al.* 2001). Cessation of milk secretion typically takes 3 days, and currently 16% of quarters become infected with new intramammary infections during this 3-day interval (Dingwell *et al.* 2002). Lactating women are currently not provided with a procedure to reduce milk secretion upon cessation of breastfeeding, and are at higher risk of mastitis due to milk stasis (Michie *et al.* 2003).

An intrinsic serotonin (5-HT) system within the mammary gland has been identified as a homeostatic regulator of lactation (Matsuda *et al.* 2004, Stull *et al.* 2007, Hernandez *et al.* 2008, Pai & Horseman 2008). The mammary glands of mice, cattle and humans express unique patterns of 5-HT receptors that vary among the species (Hernandez *et al.* 2009, Pai *et al.* 2009). The mammary glands of each species express the rate-limiting enzyme, tryptophan hydroxylase 1 (TPH1), necessary for 5-HT synthesis, and 5-HT reuptake transporter (SERT), which is responsible for the reuptake of 5-HT into the cell and its subsequent degradation into its inactive metabolite (Matsuda *et al.* 2004, Stull *et al.* 2007, Hernandez *et al.* 2009, Marshall *et al.* 2010). 5-HT is synthesized via a two-step enzymatic pathway from the amino acid L-tryptophan, with 5-hydroxytryptophan (5-HTP) as the intermediate substrate (Wang *et al.* 2002). 5-HT accelerates mammary gland involution in response to milk stasis by acting on the 5-HT₇ receptor to disrupt tight junctions (TJs), as evidenced by decreases in transepithelial electrical resistance (TEER) and the TJ proteins (Stull *et al.* 2007, Pai & Horseman 2008). Culturing of mammary epithelial cells on permeable membranes (Transwell) to monitor TJ permeability has proven to be reliable for mammary epithelial cells (Stull *et al.* 2007, Marshall *et al.* 2009). Transiently, 5-HT acts to decrease TJ permeability through protein kinase A, and sustained 5-HT causes an increase in permeability by activation of p38 MAP kinase (Pai & Horseman 2008).

The class of pharmaceutical agents belonging to the 5-HT-selective reuptake inhibitor (SSRI) family target the membrane protein SERT (SLC6A4), located on neurons at pre-synaptic terminals, axons, dendrites, neuronal cell bodies and on other cells such as platelets and mammary epithelium. By inhibiting SERT, the SSRIs increase the cellular exposure to 5-HT, and thereby the activation of 5-HT signaling pathways. SSRIs are among the most widely prescribed classes of drugs in the United States, with 232.7 million prescriptions being dispensed in 2007 (Thomas & Ellingrod 2009).

Because of their specificity for SERT, and their kinetic rather than stoichiometric mode of action, SSRIs are well tolerated in patients and the potential for toxicity in overdose is very low (Barbey & Roose 1998).

It was recently shown that there is a delay in secretory activation in mothers who take SSRIs, and initiation of local involution in mice with fluoxetine (FLX) pellets implanted in the lactating mammary gland (Marshall *et al.* 2010). The objectives of the current experiments were to 1) determine the effects of SSRI treatment on TJ permeability in bovine mammary epithelial cells (BMEC) *in vitro*, 2) determine the effects of SSRI treatment on milk protein gene expression in lactogenic primary BMEC (pBMEC) *in vitro* and 3) determine the effects of FLX and 5-HTP intramammary infusions on TJ status and milk secretion in Holstein dairy cows.

Materials and Methods

Cell culture studies

To elucidate the effects of FLX (Sigma) on TJ and milk protein mRNA expression, experiments were designed using pBMEC. Tissue dissociation and pBMEC cell isolation were performed as previously described (McGrath 1987, Hernandez *et al.* 2008).

For TJ experiments, pBMEC were plated on rat-tail collagen-coated dishes until they reached confluency (~6 days) in growth media consisting of DMEM:F12 (50:50; Invitrogen), 10% fetal bovine serum (Hyclone, Logan, UT, USA), 10 µg/ml insulin (Sigma), 25 ng/ml epidermal growth factor (EGF; Sigma), 10 ng/ml hydrocortisone (Sigma), 10 mM sodium acetate (Sigma) and 1:100 antibiotic/antimycotic (Invitrogen). At confluency, pBMEC underwent a differential trypsinization, in which cells were treated for 2 min with trypsin to remove non-epithelial cells, and then treated with trypsin for an additional 8 min to detach epithelial cells, which were plated on uncoated dishes. Upon reaching confluency on non-collagen-coated dishes, pBMEC were trypsinized, counted and then seeded at 10⁵ cells/cm² onto 0.4 µM Transwell polyester membrane supports in 12-well plates (Corning, Corning, NY, USA). Cells were fed once daily in the basal and apical chambers until plateau resistance (~2500 Ω cm²) was achieved (~6 days). TEER (Ω cm²) was measured daily prior to feeding the cells. At this time, cells were changed to medium with their respective treatments (40, 100, 200 and 400 µM FLX) in the apical chamber only for 72 h, while the basolateral chamber received media without FLX. Results are expressed as a percent change in TEER relative to the control.

For lactogenic experiments, pBMEC were cultured in 3D collagen gels (McGrath 1987, Hernandez *et al.* 2008, 2009, Stiening *et al.* 2008). Epithelial cells in the form of organoid clumps consisting of ductal and alveolar cells were isolated from four multiparous, non-lactating (~30 days dry)

Holstein cows as previously described (McGrath 1987, Hernandez *et al.* 2008, 2009). Preparation of type I collagen was performed, as previously described (McGrath 1987, Hernandez *et al.* 2008, 2009). Briefly, pBMEC were thawed, re-suspended in DMEM:F12, mixed with neutralized type I collagen at $4\text{--}6 \times 10^5$ organoids/ml of collagen mixture and cultured in 24-well plates, as previously described (Hernandez *et al.* 2008, 2009). The average DNA content of one vial of pBMEC was 486.7 ± 84.91 µg/ml (mean $\pm$ s.d.), providing sufficient cells for 72 wells in an average of ~ 6.8 µg DNA/well. pBMEC were grown for 8 days in serum-free media (10 µg/ml insulin, Sigma and 25 ng/ml EGF, Sigma) and then treated with a lactogenic hormone complex (500 µg/ml insulin; Sigma), 100 ng/ml prolactin (NIDDK, Bethesda, MA, USA) and 10 ng/ml hydrocortisone (Sigma), and gel release, with and without FLX treatments (5, 10, 20, 40, 400 µM, and 1.4 mM). Medium was exchanged every 48 h and FLX treatments were initiated at the time of lactogenic treatment of pBMEC and continued for 48 h.

In vivo study

The study was conducted from 21 to 24 December, 2009 on a 10 000 cow commercial dairy in Stanfield, AZ, USA. The University of Arizona's Institute of Animal Care and Use Committee approved all protocols and use of animals in the current study. Prior to the start of the study, all cows were being milked three times daily. Cows were fed three times daily and feed was pushed up every half hour. Diet analysis was conducted by Chandler Analytical Laboratories (Chandler, AZ, USA; Table 1). All cows were housed in open dry-lot facilities with Saudi style shades, and pens were identical in size, location and design and all dairy cows in the experiment were in late lactation, healthy and producing at least 20 kg of milk/day when assigned to the study. A total of 30 multiparous cows were identified and randomly assigned to once-a-day milking in the hospital barn using Dairy Herd Improvement Association (DHIA) test equipment meters followed by one of the two treatments for 3 days and then sealing the teats on day 4. The control (CTRL) group received intramammary infusions of the carrier (sterile water mixed with sterile mineral oil at 9:1 ratio) followed by ToDAY (cephapirin sodium; Boehringer Ingelheim, St Joseph, MO, USA) in each quarter for 2 days, and on the third day, the carrier was followed by Quartermaster (Pfizer, New York, NY, USA) and Orbaseal (Pfizer). The FLX group received 5 mg of FLX in the carrier followed by Today in each quarter for 2 days and followed by Quartermaster and Orbaseal on the third day. The 5-HTP group received 5 mg of 5-HTP in the carrier followed by Today in each quarter for 2 days followed by Quartermaster and Orbaseal on the third day. Blood samples were obtained by tail venipuncture on days 2, 3 and 4 after initiation of treatments to measure plasma FLX concentrations. All milk samples were visually inspected for clumping immediately after collection to determine the

Table 1 Diet composition for lactating Holstein dairy cattle used for experiment

Composition	C and Trt
Ingredients, % of DM	
Alfalfa hay	9.24
Haylage	3.85
Corn silage	11.08
Alfalfa green chop	10.15
Millrun	1.94
Corn (steam flaked)	16.65
Barley	24.01
Amino plus	2.53
Corn distillers grain	4.65
Whole cottonseeds	5.74
Ammoniated cottonseed	4.74
Maxxer	2.79
Molasses (cane)	4.33
Corn gluten feed	2.60
Mineral and vitamin mix	2.73
Chemical composition	
NE _L , Mcal/lb of DM	1.62
CP, % of DM	32.26
ADF, % of DM	33.18
NDF, % of DM	51.32
Ash, % of DM	16.34
Fat, % of DM	11.16
Ca, % of DM	2.10
P, % of DM	0.85
Mg, % of DM	0.67
S, % of DM	0.53
K, % of DM	2.86
Na, % of DM	1.05
Cl, % of DM	1.41
Fe, ppm of DM	590.04
Mn, ppm of DM	153.83
Cu, ppm of DM	66.90
Co, ppm of DM	4.12
Zn, ppm of DM	134.53

presence of mastitis/inflammation. Milk component analysis was conducted at Arizona DHIA, Tempe, AZ, USA. Milk fat, protein, lactose and solids-non-fat were analyzed using AOAC-approved infrared analysis (AOAC International 2007); somatic cell count (SCC) was done using AOAC-approved cell-staining techniques (AOAC International 2007). The International Dairy Federation and FDA certified all equipment used in the analyses.

All solutions were prepared using sterile-filtered mineral oil mixed with FLX (Sigma), 5-HTP (Sigma) or sterile filtered water as 10% solutions. FLX was prepared as a 5 mg/ml solution in water then sterile filtered and stored at 4 °C for the 4 days of the trial. 5-HTP was prepared daily in 0.5 M HCl and then titrated with 10 M NaOH to a final pH of 7.8 and stored at 4 °C.

Syringes were prepared using 1 ml sample in a 10 ml syringe and 9 ml mineral oil in a second 10 ml syringe connected to each other using a sterile micro-emulsifying needle (Popper & Sons, Inc., New Hyde Park, NY, USA). Samples were emulsified into a single syringe, which was

capped, covered with foil and stored at room temperature until use. Sixty-two doses of each treatment were prepared daily and were mixed within 24 h of use.

Immunofluorescent imaging

pBMEC cultured on permeable membranes (Transwell, Corning) were removed from cell culture dishes for immunostaining 48 h after the initiation of treatment and were fixed on slides by a 20 min incubation in 4% paraformaldehyde, then rinsed and stored in sterile PBS at 4 °C. Sections of permeable membranes were permeabilized in 0.1% Triton X-100 and underwent antigen retrieval in borate buffer with pH=8.5 (80 mM boric acid and 20 mM sodium borate) overnight at 75 °C. Sections were then blocked with immunofluorescent-blocking buffer (130 mM NaCl, 7 mM Na₂HPO₄, 3.5 mM NaH₂PO₄, 0.05% NaN₃, 0.2% Triton X-100, 0.05% Tween-20 and 0.1% BSA) for 1.5 h at room temperature. Sections were incubated with primary antibody (1:50, mouse anti-human, chicken, bovine, zona occludens 1 (ZO-1), Invitrogen) for 75 min at room temperature and with secondary antibody (1:1000, goat anti-mouse Alexa Fluor 488 Fab fragments; Invitrogen) for 45 min at room temperature. TOPRO-3 (Invitrogen) was used as the nuclear stain following manufacturer's instructions. Images were obtained using a Zeiss LSM510 NLO Two-Photon Microscope (Zeiss, Thornwood, NY, USA). Images were collected in both the *x-y* and *x-z* planes of the permeable membranes.

Quantitative real-time reverse transcription-PCR

Total RNA from pBMEC lactogenic cultures was isolated using Tri-Reagent per manufacturer's instructions (Molecular Research Center, Cincinnati, OH, USA). The integrity of the RNA was determined using a u.v./visible spectrophotometer (Ultraspec 3100 pro, Amersham Biosciences). For pBMEC isolated from three animals, two wells of a 24-well culture plate were pooled to represent one sample for RNA extraction, with *n* = 4/treatment. In a given experiment, each treatment was represented in each culture plate, for a total of four culture plates/experiment. The Quanti-Tect Reverse Transcription kit (Qiagen) was utilized for cDNA synthesis from purified mRNA, per manufacturer's instructions.

Quantitative real-time reverse transcription-PCR (qPCR) was conducted with the Step One Plus Real-Time PCR Detection System (Applied Biosystems, Foster City, CA, USA) using fast SYBR green master mix (Applied Biosystems) for the bovine milk protein genes α -lactalbumin and β -casein (Hernandez *et al.* 2009) per manufacturer's instructions. Ribosomal S15 served as the housekeeping gene (forward primer 5'-CGCGACATGATCATTCTAAA-3', reverse primer 5'-TTACTTGAGGGGGATGAAGC-3'). Analysis of gene expression was conducted using the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen 2001). Gene expression was quantified relative to the control (lactogenic complex + gel release) in a

respective sample. All primers met criteria for analysis by the $2^{-\Delta\Delta C_t}$ method, with efficiencies between 95 and 105% (Livak & Schmittgen 2001). Amplicons were sequenced prior to use in quantitative real-time PCR assays.

Plasma FLX assay

Plasma was collected from animals via tail venipuncture and was processed for analysis of FLX levels using a FLX ELISA (Neogen Corporation, Lexington, KY, USA), which measures the competition between FLX and its metabolite (norfluoxetine) for antibody-binding sites. Assay was performed per manufacturer's instructions.

Plasma lactose assay

Plasma collected from animals via tail venipuncture was processed and analyzed for lactose, using an enzymatic assay that measures the conversion of lactose into galactose and glucose via the enzyme lactase (Biovision, Mountain View, CA, USA). Assay was performed per manufacturer's instructions.

Na⁺ and K⁺ analysis of milk

Milk samples collected from animals on the study were analyzed for Na⁺ and K⁺ content using a digital dual-flame photometer (Cole Parmer Instrument Company, Vernon Hills, IL, USA). Samples were initially diluted in a 3 M lithium solution and 160 mmol Na⁺ and K⁺ solutions were utilized as internal standards.

Data analysis

Data analysis was conducted using Prism for Macintosh, version 5.0b for *in vitro* experiments (GraphPad Software, San Diego, CA, USA). Statistical analysis of TEER in TJ cultures was performed using a one-way ANOVA, with a Tukey's *post-hoc* test on percent change in TEER relative to control samples. Statistical analysis of qPCR data was conducted using gene expression relative to the control (lactogenic media + gel release) in a respective sample using a one-way ANOVA with a Tukey's *post-hoc* test. Graphical representation of the data is represented by expression of treatments relative to the lactogenic control ($2^{-\Delta\Delta C_t}$). The $\Delta\Delta C_t$ was calculated as ΔC_t of a respective treatment – ΔC_t of the control.

Data from the *in vivo* experiment were analyzed using ANOVA procedures of SAS (SAS Inst., Cary, NC, USA). Milk yields (recorded during the acclimation period and prior to treatments) were analyzed using the PROC MIXED procedure with repeated measures, and included as a covariate in the analysis with a Tukey–Kramer post-test and compound symmetry as the covariance structure. The treatment $\times$ day interaction was not significant (*P* = 0.64), allowing day and treatment effects to be reported independently. Dependent variables tested were milk yield, milk composition, milk Na⁺ and K⁺, plasma lactose, plasma FLX, core body temperature

Figure 1 Barrier disruption of pBMEC treated with FLX. Changes of TEER ($\Omega \text{ cm}^2$) in pBMEC treated with FLX (40, 100, 200, 400 μM and 1.4 mM) beginning after TEER measurement on day 6 for 24 h (day 7) and 48 h (day 8). Data are represented as mean $\pm$ S.E.M. The treatments resulted in significant, concentration- and time-dependent, decreases in TEER after 24 and 48 h ($P < 0.0001$).

and udder surface temperature. The independent variables included treatment, day, parity and the respective interactions. The level of significance was set at $P < 0.05$ for all main effects and interactions and the LSMEANS test was conducted when significance was detected.

Results

FLX treatment disrupted barrier function in pBMEC transwell cultures

pBMEC were cultured on permeable membranes in serum-containing medium for 6 days prior to reaching plateau

TEER ($\sim 2500 \Omega \text{ cm}^2$). Thereafter, they were treated with various concentrations of FLX for 48 h with the treatments being applied on the apical side exclusively (Fig. 1). Immunostaining confirmed that SERT was present on the apical membrane of pBMEC (Fig. 2), as was demonstrated previously in human mammary epithelial cells (Stull *et al.* 2007). FLX treatment of pBMEC resulted in a concentration-dependent decrease in TEER at 24 and 48 h (Fig. 1). This is in contrast to the biphasic response typically seen in human mammary epithelial cells cultured on selectively permeable membranes (Marshall *et al.* 2010). The 400 μM and 1.4 mM concentrations decreased TEER to a baseline level of $\sim 100 \Omega \text{ cm}^2$ after 24 h of treatment ($P < 0.0001$).

Immunofluorescent staining for the TJ protein ZO-1 was conducted on pBMEC treated with various concentrations of FLX for 48 h. Treatment with 40 and 100 μM FLX resulted in a decreased intensity of ZO-1 staining compared with the untreated control, but did not completely extinguish ZO-1 (Fig. 2A and B). However, at 200 μM , ZO-1 staining was not detectable after 48 h of treatment (Fig. 2C). Furthermore, cells treated with higher concentrations of FLX for 48 h displayed a loss of cells and fragmented nuclei, as visualized by TOPRO-3 staining, indicating cell death (Fig. 2D).

FLX decreased milk protein gene expression in pBMEC lactogenic cultures

3D collagen gel cultures of pBMEC treated with a serum-free lactogenic hormone complex in combination with gel release have been extensively used to model stage I lactogenesis (McGrath 1987, Hernandez *et al.* 2008, 2009, Stiening *et al.* 2008). FLX treatment resulted in a concentration-dependent decrease in α -lactalbumin mRNA expression relative to the lactogenic control in lactogenic pBMEC cultures ($P < 0.0001$; Fig. 3A). Exogenous FLX treatment of lactogenic pBMEC cultures resulted in a biphasic effect on

Figure 2 Effects of FLX treatment on the TJ protein, ZO-1. (A–F) Immunostaining of the ZO-1 protein (blue) and nuclei (red) in primary bovine mammary epithelial cells (pBMEC) cultured on permeable membranes treated with a range of FLX (A) Control, (B) 40, (C) 100 and (D) 200, (E) 400 μM and (F) 1.4 mM for 48 h. All images were taken at $40\times$ objective magnification using a Zeiss LSM510 NLO Two-Photon Microscope (Zeiss, Thornwood, NY, USA). (G) Immunostaining for SERT (green) and nuclei (red) in untreated pBMEC cultured on permeable membranes after 10 days in culture, taken as cross section in an x-z plane. Immunostaining indicates the presence of SERT on the apical membrane of pBMEC.

Figure 3 Effects of FLX treatment on milk protein mRNA expression in pBMEC. Mean and S.E.M. of (A) α -lactalbumin and (B) β -casein mRNA expression relative to lactogenic control in pBMEC treated with a concentration range of FLX (5 μ M–1.4 mM) plotted as mRNA expression relative to lactogenic control; significance is represented by the following: * $P < 0.05$.

β -casein mRNA expression, with increased expression at low concentrations relative to the lactogenic control ($P < 0.05$; Fig. 3B) while alternatively decreasing expression $\sim 90\%$ compared with the lactogenic control at high concentrations ($P < 0.001$; Fig. 3B).

Intramammary infusion of FLX increased TJ permeability and decreased milk yield in Holstein cows

Multiparous Holstein cows that were milked three times daily and were producing at least 20 kg of milk/day were assigned to the study. As an indicator of TJ status, milk Na^+ and K^+ concentrations and plasma lactose were measured (Stelwagen *et al.* 1999). The milk $\text{Na}^+:\text{K}^+$ ratio was increased to 2.44 in FLX-treated animals versus 1.44 in control animals in the 48 h samples ($P < 0.01$; Fig. 4A). While there was no significant change in the milk $\text{Na}^+:\text{K}^+$ ratio in the 24 h samples after FLX treatment, it is important to note that the composition of milk collected from the udder cistern primarily reflects milk that was secreted earlier and accumulated in the udder. In contrast, plasma lactose is an immediate indicator of TJ status. Plasma lactose concentrations were increased 2-fold in FLX-infused animals by 24 h post-treatment initiation ($P < 0.0001$), and remained elevated

at 48 h ($P < 0.05$, Fig. 4B). The increase in the milk $\text{Na}^+:\text{K}^+$ ratio and the increase in plasma lactose levels in FLX-treated animals support the hypothesis that one aspect of 5-HT activity leads to opening of TJs. There were no significant changes in TJ status seen in 5-HTP-treated animals (Fig. 4A and B).

Data for milk yield were analyzed by regression, with each animal serving as its own control. This approach allowed us the maximum power to detect changes over time within each group. However, for the sake of clarity, the data are represented graphically as group means and errors, rather than as individual animals (Fig. 5). The treatment effect on milk yield was highly significant by regression analysis ($P < 0.01$). At 24 h treatment, FLX-treated cows exhibited the greatest average decline in milk yield (nearly 11 kg) compared with those treated with 5-HTP (8.6 kg) or controls (7.5 kg; $P < 0.05$). Although the decline of milk yield in 5-HTP-treated cows was less rapid than in FLX-treated cows, the regression analysis indicated a significant acceleration in the 5-HTP-treated cows, compared with controls ($P < 0.05$). Milk composition was not different among any of the

Figure 4 Effects of intramammary infusions of FLX on TJ status in Holstein dairy cows. (A) Mean with S.E.M. of milk $\text{Na}^+:\text{K}^+$ ratio in milk collected from cows treated for 24 and 48 h with intramammary infusions of vehicle control (CTRL), 5 mg FLX or 5 mg 5-HTP. Significance is represented by the following: ** $P < 0.01$. (B) Mean with S.E.M. of plasma lactose (nmol) in cows treated with intramammary infusions of CTRL, 5 mg FLX, or 5 mg 5-HTP. Significance is represented by the following: * $P < 0.05$, *** $P < 0.0001$.

Figure 5 Milk production in Holstein cows treated with intramammary infusion of FLX, 5-HTP or a vehicle control. Milk yield (kg; group means $\pm$ S.E.M.) of Holstein cows treated with intramammary infusions of a vehicle control (CTRL), 5 mg FLX or 5 mg 5-HTP. The columns are labeled with the mean value for milk yield (kg) of each group. See text for full description of statistical analysis and inferences.

treatment groups (Table 2). The absence of any increase in SCC indicates that there was no observable inflammatory response to treatments. Plasma FLX concentrations were not increased until 4 days post-infusion in FLX-treated animals only (2.186 vs 0 ng/ml; $P < 0.0001$). As indicators of health status, rectal temperatures, udder surface temperatures and respiration rates were measured in all groups. Rectal temperatures were found decreased in animals receiving FLX than in controls and 5-HTP-treated animals ($P < 0.03$; Table 3). Additionally, udder surface temperatures were decreased in all groups over the treatment interval, as milk yield waned ($P < 0.001$; Table 3), and was lower in FLX-treated animals compared with controls and 5-HTP-treated cows ($P < 0.003$; Table 3). Presumably, the decline in udder surface temperature was associated with the decreasing milk synthesis and mammary blood flow, and the absence of elevated rectal or udder temperatures supports the lack of inflammatory responses to the treatments. No significant changes in respiration rates were detected.

Discussion

5-HT is an autocrine–paracrine signaling molecule, which has recently been demonstrated to accelerate the rate of mammary gland involution and regulate mammary gland homeostasis (Matsuda *et al.* 2004, Stull *et al.* 2007, Pai & Horseman 2008). While a variety of 5-HT receptors in the mammary gland of different species have been identified, the components involved in the synthesis (TPH1) of 5-HT and reuptake into the cell for degradation (SERT) are conserved (Matsuda *et al.* 2004, Hernandez *et al.* 2009, Marshall *et al.* 2010). Inhibition of SERT activity through the use of SSRIs such as FLX modifies endogenous 5-HT activity by increasing the exposure of the cell to endogenous 5-HT. Recently, it was demonstrated that treatment of human mammary epithelial cells with SSRI resulted in effects similar to those seen with exogenous 5-HT treatment (Marshall *et al.* 2010).

Disruption of TJs in the mammary gland is a hallmark of mammary gland involution. The dynamics of TJs are largely regulated by the cycle of milk accumulation and removal, with TJ permeability decreasing when milk is frequently removed and TJ permeability increasing during milk stasis (Nguyen & Neville 1999). Cultures of mammary epithelial cells on permeable membranes (Transwell) provide a reliable, consistent model for the examination of polarized mammary epithelial cells, with the measurement of the electrical conductance across the mammary epithelium (between the apical and basolateral plasma membranes), providing indications of TJ status (Stull *et al.* 2007, Marshall *et al.* 2009). Using pBMEC cultured on permeable membranes, we demonstrated that apical treatments of FLX resulted in decreases in TEER within 24 h of treatment, and that the extent of the decrease in TEER was concentration-dependent. Unlike human mammary epithelial cells, we did not see a biphasic effect of FLX on TEER. Beginning with apical treatments of 100 μ M FLX, TJs were completely disassembled after 24 h of treatment as measured by TEER and lack of peripheral immunostaining for ZO-1 (Figs 1 and 2A–D). ZO-1 is an adaptor protein required in the

Table 2 Milk composition in Holstein cattle treated with intramammary infusions of fluoxetine (FLX) and 5-hydroxytryptophan (5-HTP). Values displayed are means with S.E.M.

	Fat (%)	Protein (%)	Lactose (%)	SNF (%)	SCC (cells/ml)
24 h					
Control	4.3 (0.3)	3.4 (0.2)	4.0 (0.2)	8.5 (0.4)	576 (206)
FLX	4.8 (0.4)	3.8 (0.2)	3.7 (0.2)	8.5 (0.3)	602 (242)
5-HTP	3.6 (0.5)	3.5 (0.2)	4.4 (0.3)	8.9 (0.4)	552 (306)
48 h					
Control	3.1 (0.4)	4.0 (0.2)	3.7 (0.2)	8.7 (0.3)	489 (217)
FLX	3.5 (0.4)	3.9 (0.2)	3.9 (0.2)	8.8 (0.3)	612 (216)
5-HTP	3.9 (0.4)	4.0 (0.2)	3.8 (0.2)	8.8 (0.3)	796 (228)
72 h					
Control	5.7 (0.3)	4.2 (0.2)	2.8 (0.2)	8.3 (0.2)	852 (177)
FLX	5.2 (0.3)	3.8 (0.2)	2.9 (0.2)	8.3 (0.2)	1173 (190)
5-HTP	5.9 (0.4)	4.4 (0.2)	3.3 (0.2)	8.9 (0.3)	657 (228)

Table 3 Health parameters measured in Holstein cattle treated with intramammary infusions of fluoxetine (FLX) and 5-hydroxytryptophan (5-HTP). Values displayed are means (S.E.M.)

	Rectal temperature (°C)	Udder temperature [†] (°C)	Respiration rate (breaths/min)
24 h			
Control	38.7 (0.14)	29.4 (0.19)	35.3 (1.61)
FLX	37.8 (0.33)*	28.7 (0.26)	34.6 (1.08)
5-HTP	38.3 (0.14)	29.2 (0.26)	32.8 (1.18)
48 h			
Control	38.3 (0.15)	26.6 (0.31)	32.6 (0.92)
FLX	38.2 (0.14)	26.2 (0.28)	34.0 (1.09)
5-HTP	38.4 (0.11)	26.5 (0.31)	34.6 (1.16)
72 h			
Control	38.3 (0.10)	24.3 (0.34)	30.9 (0.98)
FLX	38.2 (0.07)	23.3 (0.38)	30.1 (0.95)
5-HTP	38.2 (0.08)	23.7 (0.69)	29.5 (1.24)

*Rectal temperatures were significantly reduced in FLX-treated animals at 24 h of treatment ($P<0.03$).
[†]Udder temperatures were significantly decreased in all treatments at 48 and 72 h of treatment ($P<0.001$).
FLX-treated animals had significantly lower udder surface temperatures than either 5-HTP or control groups at all time points ($P<0.003$).

assembly of TJs and is a common morphological marker of TJs (Paris *et al.* 2008). Additionally, as concentrations of FLX were increased, we saw a subsequent decrease in nuclei number, as well as nuclei fragmentation, indicated by TOPRO-3 staining (Fig. 2A–F). Previous reports in human and BMEC demonstrated that FLX disrupted TJs when both the apical and basolateral chambers were treated with the drug (Marshall *et al.* 2010).

Stage I lactogenesis, or secretory differentiation, begins at mid-pregnancy and involves increased and sustained expression of milk protein genes such as β -casein and α -lactalbumin, as the mammary epithelium differentiates into lactocytes (Pang & Hartmann 2007). Therefore, we utilized an *in vitro* model in which to measure the effects of FLX on the milk protein genes β -casein and α -lactalbumin (McGrath 1987, Hernandez *et al.* 2008, 2009). Upon treating lactogenic pBMEC cultures with increasing concentrations of FLX, β -casein mRNA expression responded biphasically, with substantial suppression of expression beginning at 40 μ M (Fig. 3B). This is in accordance with previous reports in which treatment of human mammary epithelial cells with exogenous 5-HT and FLX resulted in a biphasic effect on TJ permeability, with low concentrations of 5-HT potentiating TJ formation and high concentrations resulting in disassembly of TJs (Pai & Horseman 2008, Marshall *et al.* 2010). There was a concentration-dependent suppression of α -lactalbumin (Fig. 3A). α -Lactalbumin is the key protein involved in the synthesis of lactose, which is the primary osmolyte for milk secretion (Akers 2002). The ability of FLX to cause concentration-dependent inhibition of key milk protein genes suggests that increased cellular exposure to endogenously synthesized 5-HT will suppress lactation. Previous reports demonstrated that nursing mothers who are also taking SSRI treatment exhibited a delay in the onset of

lactogenesis stage II (Marshall *et al.* 2010). Additionally, it was also determined that lactating mice implanted with FLX pellets in the mammary gland exhibited a rapid local involution of the gland.

Current practices targeting mammary gland involution focus on abrupt cessation of milking in combination with antibiotic therapy to prevent mastitis infections. Previous studies in which FLX pellets implanted in mouse mammary glands resulted in increased rates of mammary gland involution (Marshall *et al.* 2010). However, pellet implantation is not a practical method that can be utilized for a dry-off treatment in lactating mothers or dairy animals. In order to determine the ability of FLX to disrupt lactation in an *in vivo* model, we infused FLX directly into the teats of lactating, multiparous Holstein cows at the end of their respective lactation cycles. Additionally, we investigated the effects of 5-HTP, which is an intermediate in the 5-HT synthesis pathway that bypasses the rate-limiting step (Wang *et al.* 2002) and was also delivered by intramammary infusion. Our hypothesis was that milk yield would decline in all animals with reduced milking stimulus, but this decline would be accelerated in FLX- and 5-HTP-treated animals. Intramammary infusions of FLX resulted in a more rapid decline in milk production (10.9 kg decrease by 24 h) than either 5-HTP-treated (8.6 kg) or control (7.5 kg) animals. Plasma lactose and the milk Na^+/K^+ ratio are typical *in vivo* measurements of TJ status in lactating animals (Stelwagen *et al.* 1999). Cows treated with intramammary infusions of FLX exhibited disruption of TJs, as indicated by increases in plasma lactose (24 and 48 h after initiation of FLX treatment) and the milk Na^+/K^+ ratio (48 h). We did not see increases in the milk Na^+/K^+ ratio by 24 h, presumably because the bulk of the milk collected from the udder cisterns had been synthesized much earlier. Intramammary infusions of 5-HTP modestly

increased the rate of milk decline ($P < 0.05$) at the dose used here. However, it did not cause measurable changes in TJ status. Future experiments should be conducted with higher doses of 5-HTP. Cows receiving the FLX treatment also displayed decreased udder and rectal temperatures compared with 5-HTP-treated and CTRL animals, although all animals exhibited decreased udder temperatures upon treatment, indicating reduced metabolism and/or blood flow and the lack of inflammatory responses. There were no increases in SCC after treatments, further indicating the lack of inflammatory responses (Table 2). The ability of FLX, and to a lesser extent 5-HTP, to accelerate the involution process in dairy animals entering the dry period could be important in preventing future mammary gland infections, either along with, or instead of, currently used methods (i.e. antibiotic treatments). Currently, treatments that rapidly terminate lactation in the dairy cattle are not in practice despite previous reports that acceleration of the involution decreases incidence of mastitis (Oliver & Smith 1982, Gulay *et al.* 2009).

The ability of an SSRI to increase the activity of 5-HT in the lactating mammary gland has recently been examined. Our data indicate that apical treatment of pBMEC cultured on permeable membranes with FLX disrupts TEER and ZO-1 staining *in vitro*. Furthermore, we demonstrate that FLX treatment decreases milk protein gene expression in a concentration-dependent manner in a serum-free lactogenic model, and finally, that intramammary infusions *in vivo* result in a greater decline in milk yield in Holstein cows. The implications of the ability of FLX to accelerate mammary gland involution will be of great importance to improving the health of animals entering the dry period portion of their respective lactation cycles. Future studies should be directed at determining the optimum conditions (i.e. concentrations, combination therapies with 5-HTP and/or antibiotics) for the use of FLX as a tool to manipulate mammary gland involution *in vivo*.

Declaration of interest

LLH, RJC and NDH declare interests in intellectual property related to this work. RJC and NDH declare interests in commercialization of related technologies through Amelgo, LLC.

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