Distribution of mRNA coding for 5-hydroxytryptamine receptor subtypes in the intestines of healthy dairy cows and dairy cows with cecal dilatation-dislocation

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Objective—To investigate the distribution of mRNA coding for 7 subtypes of 5-hydroxytryptamine receptors (5-HTRs) in the intestines of healthy dairy cows and dairy cows with cecal dilatation-dislocation (CDD).

Sample Population—Full-thickness intestinal wall biopsy specimens were obtained from the ileum, cecum, proximal loop of the ascending colon, and external loop of the spiral colon (ELSC) of 15 cows with CDD (group 1) and 15 healthy dairy cows allocated to 2 control groups (specimens collected during routine laparotomy [group 2] or after cows were slaughtered [group 3]).

Procedure—Amounts of mRNA coding for 7 subtypes of 5-HTRs (5-HT1A, 5-HT1B, 5-HT1D, 5-HT1F, 5-HT2A, 5-HT2B, and 5-HT3) were measured by quantitative real-time reverse transcriptase–PCR assay. Results were expressed as the percentage of mRNA expression of a housekeeping gene.

Results—Expression of mRNA coding for 5-HT1B, 5-HT2B, and 5-HT3 was significantly lower in cows with CDD than in healthy cows. For 5-HT2B and 5-HT3, significant differences between cows with CDD and control cows were most pronounced for the ELSC. Expression of mRNA for 5-HT1D, 5-HT1F, and 5-HT2A was extremely low in all groups, and mRNA for 5-HT1A was not detected.

Conclusions and Clinical Relevance—Relative concentrations of mRNA coding for 5-HT1B, 5-HT2B, and 5-HT3 were significantly lower in the intestines of cows with CDD than in the intestines of healthy dairy cows, especially for 5-HT2B and 5-HT3 in the ELSC. This supports the hypothesis that serotonergic mechanisms, primarily in the spiral colon, are implicated in the pathogenesis of CDD. (Am J Vet Res 2006;67:95–101)

Cecal dilatation-dislocation is one of the most economically important noninfectious diseases of the GIT in dairy cows, and in Switzerland, the prevalence is the same as for abomasal displacement in dairy cows. Clinically, CDD is characterized by reductions in appetite, milk yield, and fecal output and signs of mild colic. Dilatation of the cecum generally precedes dislocation. However, the exact pathogenesis of the disease has not been elucidated. Originally, it was believed that CDD in cattle was triggered primarily by cecal atony, which would lead to accumulation of gas and digesta and be followed by dilatation and secondary displacement. However, investigators in a study on motility of the cecum and PLAC during the recovery period after surgery for correction of CDD suggested that a motility disturbance, not located in the cecum and PLAC but more distally in the intestine (ie, in the spiral colon), may be implicated in the pathogenesis of the disease.

Serotonin (5-HT) acts via specific receptors in the wall of the GIT to affect motility. Seven families of 5-HTRs, termed 5-HT1 to 5-HT7, with more than 15 subtypes have been identified. The genetic structure has not been established for all 5-HT subtypes. The 5-HT1, 5-HT2, 5-HT3, 5-HT4, and 5-HT7 families are guanine nucleotide binding protein (ie, G protein)–coupled receptors with 7 transmembrane regions, which regulate intracellular signalling through the adenylyl cyclase or phopholipase C pathways. In contrast, 5-HT3 functions as a ligand-gated ion channel.

Serotonin receptors are distributed in numerous mammalian tissues, including the brain, the heart, and most segments of the GIT. The latter contains > 80% of the total 5-HT, most of which is in enterochromaffin cells of the intestines but which also is found in enteric neurons. In the intestines, 5-HT acts as a neurotransmitter and as a paracrine-signalling molecule and participates in mucosal sensory transduction. Of the 5-HT subtypes identified so far, 5-HT2A, 5-HT2C, 5-HT3, 5-HT4, 5-HT5, 5-HT6, and 5-HT7 primarily modulate GIT motility. The various effects induced by 5-HT depend primarily on the 5-HT involved as well as the species and tissues.
Few investigations have addressed the role of 5-HT in the regulation of GIT motility in ruminants, with most investigations conducted in rodents or humans. Studies on the pharmacologic aspects of the phasic and tonic motor events in the GIT of ruminants have revealed that cholinergic, adrenergic, serotoninergic, opiate, and dopaminergic mechanisms appear to be involved in the regulation of a wide range of physiologic functions. In sheep, duodenal distention causes inhibition of forestomach and abomasal motility via 5-HTR$_{1A}$, 5-HTR$_{1D}$, and 5-HTR$_{4}$ subtypes. The 5-HTR$_{4}$ subtype also appears to play an important role in the control of forestomach motility in sheep as well as in the initiation of migrating myoelectric complexes. Administration of 5-HT inhibits the primary cycles of the reticulo rumen in goats and sheep. Furthermore, investigators reported a role of 5-HT in the control of forestomach and antroduodenal motility in sheep. Thus, serotonin has several functions in the regulation of cyclic motor events in the GIT of ruminants, but the precise role played by specific 5-HTR subtypes is not known.

The expression of mRNA coding for 5-HTR subtypes in the GIT of healthy dairy cows has been described. In that study, investigators reported that relative concentrations of mRNA coding for 5-HTR subtypes vary among receptor subtypes and among locations of the GIT.

The objective of the study reported here was to investigate the expression of mRNA coding for receptor subtypes 5-HTR$_{1A}$, 5-HTR$_{1B}$, 5-HTR$_{1D}$, 5-HTR$_{1F}$, 5-HTR$_{2A}$, 5-HTR$_{2B}$, and 5-HTR$_{4}$ in intestinal tissues of cows with naturally developing CDD, compared with results obtained for healthy dairy cows. We hypothesized that the amount of mRNA coding for 5-HTR subtypes would differ between tissues from healthy dairy cows and cows with a motility disorder leading to CDD. Confirmation of this hypothesis may indicate involvement of 5-HTR subtypes in the pathogenesis of CDD, leading to new perspectives for pharmacologic and therapeutic interventions.

Materials and Methods

Animals—Thirty cows were included in the study; they were allocated to 3 groups. Group 1 comprised 15 dairy cows (lactating and nonlactating) examined at the Clinic for Ruminants, Vetsuisse Faculty of Berne, Switzerland for treatment of spontaneous CDD. Groups 2 and 3 consisted of healthy dairy cows. Group 2 comprised 7 healthy lactating dairy cows involved in another research project at the Clinic for Ruminants, and group 3 comprised 8 healthy dairy cows that had been sold for slaughter. The project was approved by the Swiss Board for Animal Welfare and Protection.

Sample collection—For group 1, full-thickness samples of the intestinal wall were collected during routine surgery for correction of CDD. Surgery was performed in accordance with an established method by use of laparotomy via the right flank with the cows in a standing position. After routine enterotomy of the cecum and drainage of cecal contents, the ileum (40 cm orad of the ileocecal valve), cecal body (midway between the ileocecal valve and apex of the cecum), PLAC (40 cm aborad of the ileocecal valve), and ELSC were sequentially exteriorized through the incision. A full-thickness biopsy specimen (approx 0.5 cm$^2$) was dissected from each anatomic site, and the GIT wall was closed by use of 2 inverting Cushing sutures of polyglyconate 3-0. Samples were immediately rinsed with ice-cold PBS solution (pH 7.4), stored in 2 mL of RNA stabilization solution at 4°C for 24 hours, and then frozen at –20°C until assayed. The abdomen was closed in a routine manner. Postoperative care consisted of administration of an antimicrobial for 5 days, and perfusions and spasmodyltics were administered as needed. Cows were discharged from the clinic to their owners after recovery.

For group 2, samples were collected during routine laparotomy performed in healthy dairy cows as part of another research project. The same procedure described for cows of group 1 was used for cows of group 2. Postoperative care consisted of administration of an antimicrobial for 5 days.

Tissue samples were collected for the cows of group 3 from the same intestinal locations as those for groups 1 and 2. Samples were collected within minutes after healthy dairy cows were stunned during slaughter at a local slaughterhouse. From each tissue specimen (approx 0.5 cm$^2$) was immediately dissected. Samples were rinsed with PBS solution and stored in RNA stabilization solution as described for groups 1 and 2.

Experimental protocol—Tissue samples of all groups were handled in accordance with the same protocol. Total cellular RNA from the specimens was isolated by homogenizing tissues for 5 minutes by use of a glass bead cell disrupter in 1 mL of extraction solution (80% to 100% of tissue, with a total amount of tissue of 180 to 200 mg. Samples were then incubated for 10 minutes at 23°C. After addition of 200 μL of chloroform/ml of extraction solution, samples were vortexed for 15 seconds. Tissue homogenates were then allowed to remain at 23°C for 10 minutes, which was followed by centrifugation at 12,000 × g for 15 minutes at 4°C. The RNA in the upper aqueous phase (approx 400 μL) was transferred to 1.5-mL tubes, precipitated by the addition of 500 μL of iso-2-propanol, incubated for 15 minutes at 23°C, and centrifuged at 12,000 × g for 10 minutes at 4°C. Supernatant was decanted, and RNA pellets were washed twice with 75% ethanol; each washing was followed by centrifugation at 9,200 × g for 8 minutes at 4°C. Pellets were then completely dried during 3 minutes at 23°C and diluted in 25 μL of RNase-free water. Total extracted RNA was quantified by UV spectroscopy at an optical density of 260 nm, and the stock solution was diluted by the addition of RNase-free water to create a working solution with a concentration of 100 ng/μL. Quality of recovered RNA was judged to be acceptable when the ratio for the optical density at 260 nm to the optical density at 280 nm was > 1.9.

Synthesis of first-strand cDNA was performed, as described elsewhere, with 200 units of reverse transcriptase and 100 pmol of random hexamer primers. The final concentration of cDNA was 25 ng/μL. The primer (forward and reverse) sequences of various receptor subtypes (ie, 5-HTR$_{1A}$, 5-HTR$_{1B}$, 5-HTR$_{1D}$, 5-HTR$_{1F}$, 5-HTR$_{2A}$, 5-HTR$_{2B}$, and 5-HTR$_{4}$) were obtained from another report. A master mixture was prepared to achieve the desired end concentrations (0.4 μL of H$_2$O, 1.2 μL of MgCl$_2$ [4 mM], 0.2 μL of forward primer [4 pmol], 0.2 μL of reverse primer [4 pmol], and 1.0 μL of DNA binding dye). Nine microliters of the master mixture was placed in the glass capillaries, and 1 μL of reverse-transcribed RNA was added as a template for the PCR assay. Product-specific PCR cycle conditions for all receptor subtypes and the housekeeping gene GAPDH were as described elsewhere. After the last amplification cycle,
PCR products were examined by use of a melting curve analysis to ensure specific products had been amplified. Quantification of mRNA coding for the housekeeping gene GAPDH was used as a reference for adjustment of gene expression in the investigated cDNA samples. Amounts of GAPDH were determined for each cow and each tissue (ie, for each sample). Expression of mRNA for each 5-HTR was reported as a percentage of mRNA expression for GAPDH and was calculated by use of the following equation:

\[
\frac{1}{2^{(C_{\text{GAPDH}} - C_{\text{5-HTR}})}} \times 100
\]

where \(C\) (5-HTR) is the crossing point for 5-HTR amplification and \(C\) (GAPDH) is the crossing point for GAPDH amplification.39

Reverse-transcribed RNA of pooled brain tissues (cortex, thalamus, and hypothalamus) from healthy dairy cows was added to each PCR assay to monitor assay-to-assay variation (ie, interassay control samples).

**Statistical analysis**—For all parametric statistical analyses, the mRNA expression of each receptor subtype was logarithmically transformed to obtain a normal distribution. Correctness of the transformations was verified by use of quantile-versus-quantile plots and Lilliefors tests. To compare the logarithm of expression among various tissues and groups, a 2-factor ANOVA was performed for each receptor subtype with repeated measurements on 1 factor (ie, tissue) and fixed effects on the other factor (ie, group). The general linear model approach was used in combination with weighted least squares. Weighted values were used to correct for the unequal variances that were observed for the 3 levels of group. Fit of the models was evaluated by plotting the residuals versus the fitted values and by use of quantile-versus-quantile plots of the residuals. Factor levels were compared by calculating the difference of the corresponding factor effects and corresponding SDs. These values were then used to perform a t test. For each tissue, the logarithm of the expression for group was evaluated by a single-factor ANOVA. The degrees of freedom were adjusted in accordance with the method of Welch because the variances of the 3 levels of group were again unequal. Pairwise comparisons were then performed by use of t tests with nonpooled SDs. Significance was defined as values of \(P < 0.05\). For all statistical analyses in which multiple testing was necessary, values of \(P\) were adjusted in accordance with the method of Holm. Commercially available software packagesi,j were used for computations.

Detection limit of the real-time reverse transcriptase PCR assay is a \(C\) value of approximately 33 (ie, 33 amplification cycles until PCR products become detectable).40 Only results with \(C\) values < 33 were included in statistical analyses. Values of \(C\) < 33 are usually linearly proportional to the

<table>
<thead>
<tr>
<th>Location</th>
<th>Receptor subtype</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
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<tr>
<td>Ileum</td>
<td>5-HTR(_{1A})</td>
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<td>ND</td>
<td>ND</td>
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<td>0.0030–0.0046</td>
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<td>2.21–3.38</td>
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<td>1.02–4.77</td>
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<td>ND</td>
<td>ND</td>
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<td>5-HTR(_{1D})</td>
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<td>0.0009–0.0136</td>
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<td>5-HTR(_{4})</td>
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<td>1.58–3.01</td>
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<td>0.0083–0.0599</td>
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<td>ND</td>
<td>ND</td>
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<td>0.68–3.35</td>
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<td>0.0050–0.0320</td>
<td>0.0025–0.0083</td>
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<td>0.0111–0.0227</td>
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<td></td>
<td>5-HTR(_{4})</td>
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<td>1.94–3.26</td>
<td>2.06–3.54</td>
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<tr>
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<td>5-HTR(_{4})</td>
<td>0.25–0.76</td>
<td>0.66–2.72</td>
<td>0.56–2.60</td>
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</tbody>
</table>

Values are expressed as interquartile ranges (25th to 75th percentile) for the percentage of each 5-HTR expression in relation to expression for the housekeeping gene GAPDH. Group 1 comprises 15 dairy cows with CDD. Group 2 comprises 7 healthy dairy cows from which samples were obtained during laparotomy. Group 3 comprises 8 healthy dairy cows from which samples were obtained after stunning at a slaughterhouse.

ND = Not detected.
number of gene copies in an inverse-logarithmic manner, whereas detection of gene copies is inconsistent above this limit (ie, results become unreliable).

Results

Relative amounts of mRNA measured were extremely low for all 5-HTR subtypes in all tissues and for all groups of cows (Table 1). In all samples for all groups, amounts of mRNA were especially low for 5-HTR1F, 5-HTR1D, and 5-HTR2A and relatively high for 5-HTR1B, 5-HTR2B, and 5-HTR4 (Figure 1). We did not detect mRNA coding for 5-HTR1A in any sample.

Statistical analysis for 5-HTR1B expression revealed a significant effect for group \((P < 0.001)\) and tissue \((P = 0.022)\). Relative mRNA expression for 5-HTR1B was significantly \((P < 0.001)\) lower in cows with CDD than in the 2 control groups (Figure 1); expression did not differ significantly between groups 2 and 3. For all groups, mRNA expression for 5-HTR1B was significantly lower in the ileum than in the cecum and PLAC. There was not a significant interaction between groups and tissues. Follow-up analysis for each tissue confirmed significantly lower expression of mRNA in cows with CDD, compared with values for the 2 control groups, for all locations except for the comparison between groups 1 and 2 in the ileum \((P = 0.088)\) and ELSC \((P = 0.055); Figure 2\).

Statistical analysis for 5-HTR2B expression revealed a significant \((P < 0.001)\) effect for group. Expression of mRNA coding for 5-HTR2B was lower in cows with CDD than in healthy cows of group 3 (Figure 1). No difference was evident between cows with CDD and healthy cows of group 2 (ie, healthy cows from which samples were obtained during laparotomy) or between the 2 control groups (ie, groups 2 and 3). We did not detect a significant effect of tissue or a group-by-tissue interaction for the 5-HTR2B sub-
type. Follow-up statistical analysis between group 1 and 3 for each location revealed significant differences for the ileum (P = 0.003), PLAC (P = 0.001), and ELSC (P = 0.010) but not the cecum (Figure 2). Follow-up statistical analysis between groups 1 and 2 for each location revealed no significant differences (P= 0.053 in the ELSC). For all locations, no significant differences were evident between groups 2 and 3.

Tissue effects were significant (P < 0.001) for 5-HTR4 expression. For all groups, expression of mRNA coding for 5-HTR4 was lower in the ELSC than in the ileum, cecum, and PLAC. We did not detect significant differences among groups in the overall analysis, but a significant group-by-tissue interaction was detected. Follow-up statistical analysis for each location revealed that the relative expression of mRNA coding for 5-HTR4 in the ELSC was lower for cows with CDD than for cows in group 2 (P = 0.042) or 3 (P = 0.037), but no significant differences were evident between the 2 control groups (Figure 2). We did not detect significant differences among groups for the other locations (ie, ileum, cecum, and PLAC).

Relative expression of mRNA coding for 5-HTR1D, 5-HTR1F, and 5-HTR2A was extremely low (CP values ranging from 33 to 37 cycles). Therefore, no statistical analysis was performed for these receptor subtypes.

Discussion

The study reported here revealed differences in relative mRNA expression of 5-HTR1B, 5-HTR2B, and 5-HTR4 between cows with CDD and healthy dairy cows, which may indicate an involvement of these receptor subtypes in the pathophysiological processes of the disease.

The mRNA coding for 5-HTR4 was expressed in moderate to high amounts in all groups, and significant differences in relative expression were evident between group 1 and the control groups. Expression for group 1 (cows with CDD) was lower than for the 2 control groups. The 5-HTR4 subtype is mainly associated with decreased release of neurotransmitters (acetylcholine or 5-HT). Thus, decreased expression of mRNA coding for 5-HTR4 may indicate that, for the pathologic condition of CDD, the inhibition mediated by this receptor subtype is decreased, possibly resulting in an increase in neurotransmitter concentrations. However, it is difficult to interpret this result because the exact physiologic role of 5-HTR4 in the GIT remains unknown, especially in cattle. To interpret this result in terms of physiologic relevance, additional studies will be necessary to evaluate whether these differences in mRNA expression also reflect differences between healthy cows and cows with CDD at the protein level and with regard to function of these receptors.

Expression of mRNA coding for 5-HTR3B was higher in samples obtained from healthy dairy cows at the slaughterhouse than in cows with CDD. Amount of mRNA expression for 5-HTR3B in group 2 (samples obtained from healthy dairy cows during laparotomy) was between that of the 2 other groups; thus, we did not detect significant differences between group 2 and the other groups. This result may in part be explained by the high variability observed in data for group 2, most notably for samples from the ileum.

Statistical analysis for each location confirmed results of the overall analysis for the ileum and PLAC (ie, a marked difference between groups 1 and 3 and no difference between group 2 and either of the other groups). No significant differences were evident among groups for the cecum, probably as a result of high variability of the data. In the ELSC, groups 1 and 3 also differed significantly. Values did not differ significantly between the control groups. There was a nonsignificant (P = 0.053) difference between groups 1 and 2 for the ELSC. Thus, the expression of mRNA coding for 5-HTR3B was lower in cows with CDD, compared with expression for healthy cows of the control groups, although not significantly so between groups 1 and 2. The fact that the results of separate comparison of each of the 2 control groups with the CDD group did not result in the same conclusions (significant differences between groups 1 and 3 for the ileum, PLAC, and ELSC but no significant differences between groups 1 and 2) was not expected. The differences between groups 1 and 3 may be attributable, at least in part, to the fact that samples were collected during laparotomy from cows of group 1 and after stunning during slaughter from cows of group 3. However, the difference between groups 1 and 2 (P = 0.053) for the ELSC suggests that CDD is accompanied by a tissue-specific downregulation of mRNA expression for 5-HTR3B in the ELSC because samples were collected from cows of groups 1 and 2 by use of the same procedure (ie, biopsy of the intestinal wall during laparotomy). Thus, the lower expression of mRNA coding for 5-HTR3B in cows with CDD, compared with expression in healthy cows, is believed to be related to the disease, at least in the spiral colon.

In humans, 5-HTR3B mediates smooth muscle contraction in the ileum and colon. In mice, 5-HTR3B possibly mediates trophic functions of serotonin in the intestines. Activation of 5-HTR3B expressed on the endothelium of pigs and rats leads to relaxation of blood vessels by stimulating the release of Nitric oxide. Because mRNA was extracted from full-thickness specimens of the intestinal wall in the study reported here, it is not possible to determine the cells or structures of the intestines in which expression of mRNA coding for 5-HTR3B is modified in cows with CDD, compared with expression for healthy cows.

Effects of the 5-HTR4 subtype on the GIT of various species have been described by many authors. This subtype mediates contraction or relaxation depending on tissue location and species. In guinea pigs, 5-HTR4 mediates contraction of the stomach, ileum, and colon through the release of acetylcholine in the myenteric plexus. In contrast, in the colon of humans and esophagus and ileum of rats, 5-HTR4 located on smooth muscle cells mediates relaxation. This receptor also plays a role in secretion of chloride ions in the colon of rats and jejunum, ileum, and colon of humans. In the study reported here, amounts of mRNA coding for 5-HTR4 did not differ significantly between healthy cows or cows with CDD in the overall statistical analysis, but a tissue effect and, more importantly, a tissue-by-location interaction were detected. Follow-up statistical analysis for each loca-
tion revealed significant differences among groups for the spiral colon (ie, lower expression of mRNA coding for 5-HTR, in the CDD group, compared with expression in each of the 2 control groups). These results may support those of a study in which a motility disorder distal to the cecum and PLAC (ie, in the spiral colon) was believed to be involved in the pathogenesis of CDD.

Although a real-time reverse transcriptase–PCR assay is the technique of choice for measurements of mRNA in low abundance, the amounts of mRNA coding for 5-HTR, 5-HTRI, and 5-HTR measured in the study were less than the reliable limit of quantification. Thus, use of this kinetic PCR method in the study reported here did not allow us to gain more insight into mRNA expression of these 3 receptor subtypes in the intestinal tract of healthy cows and cows with CDD.

We did not detect mRNA coding for 5-HTRI in any sample of the GIT in any group. However, expression of this receptor has been reported in the bovine CNS by use of the same primer in another study and in interassay control samples in the study reported here. Although 5-HTRA has been identified in the GIT of several other species, this 5-HTR subtype appears to be lacking in healthy dairy cows and cows with CDD. This finding is in accordance with the results of a study in healthy animals.

We conclude from the results of the study reported here that relative expression of mRNA coding for 5-HTR in the intestines of cows with CDD differs from that of healthy dairy cows. Differences depend on receptor subtype and location in the GIT. Significant differences were found for 5-HTRI, 5-HTRII, and 5-HTR, especially in the ELSC. These differences in mRNA expression in healthy cows and cows with CDD reflect dynamic control of the 5-HTR genes. However, additional investigations are needed to determine the implications of these differences from a functional standpoint (ie, in regard to the pathophysiologic processes of CDD). Because mRNA expression does not necessarily imply functional receptors on the membrane of cells, radioligand binding studies or functional assays are warranted on the basis of the results reported here.

Decreased expression of mRNA coding for 5-HTR in the spiral colon of cows with CDD is of particular interest because this receptor subtype is one of the most important 5-HTRs with regard to modulation of GIT functions. Furthermore, agonists of this receptor subtype have been widely used as prokinetics in humans and domestic animals and may be of interest for future studies aimed at improving medical management of cows with CDD. In addition, the fact that expression of mRNA coding for the receptors was primarily modified in the spiral colon but not in the ileum, cecum, or PLAC further supports the hypothesis that the spiral colon may play an important role in the pathogenesis of CDD.

References

23. Borman RA, Burleigh DE. Human colonic mu cosa possess-


