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Quantitative mRNA Analysis of Eight Bovine 5-HT Receptor Subtypes in Brain, Abomasum, and Intestine by Real-Time RT-PCR

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ABSTRACT

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Serotoninergic pathways are involved in economically important bovine gastrointestinal (GI) motility disorders such as displaced abomasum and cecal dilatation/dislocation. The existing research tools to investigate the role of serotoninergic pathways in such disorders in ruminants comprise functional pharmacological methods, e.g., in vitro contractility studies in tissue baths, and electromyographical recordings in vivo. However, no tools for quantification of bovine serotonin receptor [5-hydroxytryptamine receptor (5-HTR)] expression were available so far. This study aimed to develop real-time RT-PCR assays for quantitative mRNA analysis of bovine 5-HTR subtypes. Because the bovine 5-HTR coding sequences (CDSs) were completely unknown, multiple species (human, mouse, and rat) alignment of complete CDS was used for primer design in highly homologous regions. LightCycler real-time RT-PCR

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assays (partial CDS) for the following bovine 5-HTR subtypes were developed and validated: 5-HTR$_{1A}$, 5-HTR$_{1B}$, 5-HTR$_{1D}$, 5-HTR$_{1F}$, 5-HTR$_{2A}$, 5-HTR$_{2B}$, 5-HTR$_{2C}$, and 5-HTR$_{4}$. Intra- and inter-assay coefficients of variation (CV) for the eight established assays were small, ranging from 0.49% to 2.46%. As a first physiological application, 5-HTR mRNA expression levels were measured in brain, abomasum, and intestine of 10 healthy, lactating dairy cows. The 5-HTR expression was quantified by normalization to the housekeeping gene glyceraldehyde-phosphate-dehydrogenase (GAPDH). The 5-HTR subtype expression levels ranged from 0.001% (5-HTR$_{2C}$ in intestine) to 1% 5-HTR/GAPDH (5-HTR$_{1B}$ and 5-HTR$_{4}$ in intestine). There were high variations of 5-HTR subtype mRNA expression within tissues across receptor subtypes and within receptor subtypes across tissues. In conclusion, accurate real-time RT-PCR assays for quantitative analysis of bovine 5-HTR subtype gene expression were developed and validated.

Key Words: Serotonin receptor (5-HTR); Bovine; Quantitative mRNA analysis; Real-time RT-PCR.

INTRODUCTION

Serotonin [5-hydroxytryptamine (5-HT)] is involved in a wide range of physiological functions as well as in a wide range of pathological states. Serotonin was first discovered as an important brain neurotransmitter that has effects on complex behaviors such as mood and appetite and that is relevant to depression, migraine, and several neuropsychiatric illnesses (1,2). Hence, 5-HT receptors (5-HTR) are best described in the central nervous system (3). However, 5-HT also plays important roles in other tissues. About 95% of 5-HT is found in the gastrointestinal (GI) tract, especially in enterochromaffin cells, but also in serotoninergic neurons (4). The 5-HT released by mechanical or vagal stimulation binds to 5-HTR and then acts locally to regulate GI function such as motility of the GI tract, which is either enhanced or inhibited via multiple 5-HTR subtypes. The 5-HTRs have been found along most segments of the GI tract of mammals (4). Although 5-HTRs have been highly conserved throughout evolution, as evidenced by molecular cloning (5), there seem to exist important differences among species in the function of individual receptor types (6,7). Fourteen different 5-HTR subtypes are known so far; they consist of at least two distinct types of molecular structures: G protein-coupled receptors (5-HTR$_{1}$, 5-HTR$_{2}$, 5-HTR$_{4}$, 5-HTR$_{5}$, 5-HTR$_{6}$, 5-HTR$_{7}$) and ligand-gated ion channels (5-HTR$_{3}$) (5). The G protein-coupled 5-HTRs regulate two major intracellular second messenger pathways, adenylate cyclase and phopholipase C (8). Receptors of the 5-HTR$_{1}$ family are negatively coupled to adenylate cyclase activation, whereas 5-HTR$_{2}$ are positively coupled to phospholipase C, and 5-HTR$_{4}$ positively coupled to adenylate cyclase activation. Thus, different cascades of intracellular events are activated via second messengers (cyclic nucleotides after activation of adenylate cyclase, or hydrolysis products of phosphoinositol in the case of phospholipase C, respectively) after interaction of serotonin with receptors (1,8,9).

In ruminants, disorders of GI motility such as displaced abomasum and cecal dilatation/dislocation are frequent and economically important (10,11). However, the serotoninergic pathways were predominantly studied in humans and rodents. The 5-HT was shown to be involved in the initiation and regulation of the myoelectric migrating complex (MMC), in the peristaltic reflex, in the regulation of stomach, small intestine and colon motility, as well as in intestinal secretion (4,9,12–23). The mechanisms of action of

5-HT in the digestive tract are not completely understood and the role of 5-HTR subtypes in the regulation of GI motility differs among species and locations along the GI tract (9,24,25).

In ruminants, 5-HT has been shown to be involved in the regulation of forestomach, abomasal, and intestinal motility (21,26–30). Most of the research available on the effects of 5-HT on GI motility in ruminants has been conducted in sheep. In this species, 5-HT reduces forestomach motility and increases the rumen wall tone (26,29,30). Similar results have been observed in goats (31). In another study in sheep, 5-HTR$_{1A}$, 5-HTR$_2$, 5-HTR$_3$, and 5-HTR$_4$ are reported to be involved in the regulation of forestomach motility occasionally recorded concomitantly to duodenal phase III activity (21,32). Furthermore, 5-HT is also implicated in the regulation of MMC patterns in the abomasum and proximal small intestine (17,19–21,27,33), but little is known about the role of the respective 5-HTR subtypes involved in motility control in ruminants.

The literature on the role of 5-HT in the regulation of motility in the bovine GI tract is almost inexistent, and, with the exception of one publication stating that 5-HT containing cells are more numerous in the bovine small intestine than in the abomasum or in the large intestine (34), we are not aware of reports on the distribution and physiological role of the different 5-HTR subtypes in the bovine digestive tract.

Quantitative real-time RT-PCR analysis is a useful tool to measure specific receptor gene expression and to differentiate between various receptor subtypes or splice variants in disease models (35–38). While the human, rat, and mouse complete coding sequences (CDS) of the 14 different 5-HTR subtypes have been determined and published, the bovine 5-HTR nucleotide sequences were completely unknown so far. However, quantitative mRNA analysis of bovine 5-HTR subtypes would be an important tool to study 5-HTR-mediated GI diseases in farm animals and could complete the present panel of research tools such as functional pharmacological methods, e.g., in vitro contractility studies in tissue baths, and electromyographical recordings in vivo.

Based on such observations, we aimed to develop LightCycler real-time RT-PCR protocols for quantitative analysis of bovine 5-HTR subtype mRNA and to compare the expression levels of bovine 5-HTRs between tissues from brain, abomasum, and intestine. We were especially interested in 5-HTR$_1$, 5-HTR$_2$, and 5-HTR$_4$ receptor subtypes, as these are expected to play most likely an important role in GI motility (4,17,21).

MATERIAL AND METHODS

Tissue Samples, Total RNA Preparation, and cDNA Synthesis

Tissue samples of brain [cortex (lobus piriformis), thalamus, and hypothalamus], abomasum [fundus, corpus, antrum pylori], and intestine [ileum, caecum, proximal loop of the ascending colon (PLAC), and spiral colon] of 10 healthy lactating cows culled in the slaughterhouse of Berne, Switzerland, were taken within 20 min of stunning. Abomasum and intestine tissue were dissected in the slaughterhouse. The heads of the cows were transferred to the Faculty of Veterinary Medicine, University of Berne, Switzerland (10 min drive), and were dissected in the high-security facilities of the Institute of Virology and the Institute of Neurology. The dissected tissues were rinsed with ice-cold PBS

(pH 7.4), kept in 3 parts of RNAlater® (Ambion Inc., Austin, TX) at 4°C for 24 h, and then stored at −20°C until assayed within 4 weeks. For RNA preparation, 200 mg of each tissue were homogenized with an Ultra-Turrax® T 8 homogenizer (IKA Werke GmbH & Co. KG, Staufen, Germany) in Sarstedt tubes using 2 mL TriFast Isolation Reagent (PeqLab Biotechnologie GmbH, Erlangen, Germany) and incubated for 5 min at room temperature (rt). After addition of 0.4 mL chloroform and vortexing for 15 s, the tissue homogenate was incubated for 10 min at rt. After centrifugation for 15 min at 12,000*g* and 4°C, the RNA (being in the upper aqueous phase) was carefully pipetted into 1.5 mL Eppendorf tubes, precipitated by adding 0.5 mL 2-propanol, and centrifuged for 10 min at 12,000*g* at 4°C. The supernatant was decanted and the RNA pellets were washed twice with 75% ethanol followed at each time by centrifugation at 9200*g* for 8 min at 4°C. The supernatant was decanted completely and after the second washing step the pellets were dried for 10 min at 37°C. The pellets were diluted in 30 μL RNase-free water.

In order to quantify the extracted total RNA, the optical density of the RNA stock solution was determined at 260 nm. Additionally, the optical density of the OD_{260nm}/OD_{280nm} (nucleic acid/protein) absorption ratio was measured which lay in an optimum range between 1.8 and 2.0. The stock solution was diluted into a working solution of 100 ng/μL by adding RNase-free water and the RNA density was again optically determined in triplicates at 260 nm.

Synthesis of first strand complementary DNA (cDNA) was performed with 200 units of reverse transcriptase (MMLV-RT, Promega, Madison, WI) and 100 pmol random hexamer primers (MBI Fermentas, St. Leon-Rot, Germany). The final concentration of reversely transcribed total RNA (cDNA) was 25 ng/μL.

For further analysis, in each individual animal the cDNA was pooled in equal amounts as described below:

- Brain pool (CNS): cortex (lobus piriformis), hypothalamus, thalamus.
- Abomasum pool: fundus, corpus, antrum pylori.
- Intestine pool: ileum, caecum, PLAC, spiral colon.

Multiple Species Primer Design

The primers used for the production of recDNA were derived either from human, rat, or mouse sequences. Primers were designed to produce an amplification product which spanned at least two exons in the highly conserved coding region of the appropriate CDS of multiple species. Therefore, a multiple CDS alignment (clusteral alignment in HUSAR® software) of the available mRNAs was done at DKFZ (http://genome.dkfzheidelberg.de/biounit/). Primer design and optimization was done in the high homology regions of the multiple alignment with regard to primer dimer formation, self-priming formation and primer melting temperature (HUSAR® software at DKFZ). Housekeeping gene oligonucleotide sequences were taken from earlier publications (38,39). The sequences of PCR forward (f) and reverse (r) primers, position of the primers in the CDSs, PCR product length, and National Center for Biotechnology Information (NCBI) accession numbers of the published nucleic acid sequences (http://www.ncbi.nlm.nih.gov/Entrez/index.html) used for primer design are summarized in Table 1.

Table 1. Sequences of PCR primers, position of the forward (f) and reverse (r) primers in CDS, PCR product lengths, and NCBI accession numbers (ac. no.) of published nucleic acid sequences used for primer design.

Primer	Sequence (5′–3′)	CDS	Length	NCBI ac. no.[a]
5-HTR_{1A} **f**	TCAGCTACCAAGTGATCACCTCT	98–120	211	XM_003692 (human)
5-HTR_{1A} **r**	GTCCACTTGTTGAGCACCTG	308–289		
5-HTR_{1B} **f**	TGCTCCTCATCGCCCTCTATG	665–685	259	XM_004117 (human)
5-HTR_{1B} **r**	CTAGCGGCCATGAGTTTCTTCTT	923–901		
5-HTR_{1D} **f**	CCTCCAACAGATCCCTGAATG	44–64	359	NM_000864 (human)
5-HTR_{1D} **r**	CAGAGCAATGACACAGAGATGCA	402–380		
5-HTR_{1F}-2 **f**	TGTGAGAGAGAGCTGGATTATGG	252–272	248	NM_000866 (human)
5-HTR_{1F}-1 **r**	TAGTTCCTTGGTGCCTCCAGAA	499–478		
5-HTR_{2A}-2 **f**	AGCTGCAGAATGCCACCAACTAT	311–333	322	NM_000621 (human)
5-HTR_{2A}-3 **r**	GGTATTGGCATGGATATACCTAC	632–610		
5-HTR_{2B}-2 **f**	AAACAAGCCACCTCAACGCCT	756–776	411	XM_048724 (human)
5-HTR_{2B}-1 **r**	TCCCGAAATGTCTTATTGAAGAG	1166–1144		
5-HTR_{2C}-2 **f**	TTCTTAATGTCCCTAGCCATTGC	280–302	251	NM_000868 (human)
5-HTR_{2C}-3 **r**	GCAATCTTCATGATGGCCTTAGT	530–508		
5-HTR_{4}-2 **f**	ATGGACAAACTTGATGCTAATGTGA	1–25	220	XM_052171 (human)
5-HTR_{4}-2 **r**	TCACCAGCACCGAAACCAGCA	220–200		
GAPDH-2 **f**[b]	GTCTTCACTACCATGGAGAAGG	265–286	197	U85042 (bovine)
GAPDH-2 **r**[b]	TCATGGATGACCTTGGCCAG	461–442		
UbC-3 **f**[c]	AGATCCAGGATAAGGAAGGCAT	86–107	654	Z18245 (bovine)
UbC-3 **r**[c]	GCTCCACCTCCAGGGTGAT	739–721		
18S-2 **f**[d]	GAGAAACGGCTACCACATCCAA	55–76	337	AF176811 (bovine)
18S-2 **r**[d]	GACACTCAGCTAAGAGCATCGA	391–370		

[a] Web address: http://www.ncbi.nlm.nih.gov/entrez/index.html.
[b] GAPDH: glyceraldehyde-phosphate-dehydrogenase.
[c] UbC: ubiquitin.
[d] 18S: 18S ribosomal RNA gene.

Quantification by Real-Time RT-PCR

Polymerase chain reaction was performed in the LightCycler® (Roche Diagnostics, F. Hoffmann-La Roche Ltd, Basel, Switzerland) with 25 ng reversely transcribed total RNA (25 ng/μL). A master-mix of the following reaction components was prepared to the indicated end concentration: 6.4 μL water, 1.2 μL $MgCl_2$ (4 mM), 0.2 μL forward primer (4 pM), 0.2 μL reverse primer (4 pM) and 1.0 μL LightCycler Fast Start DNA Master SYBR Green I® (Roche Diagnostics, F. Hoffmann-La Roche Ltd, Basel, Switzerland). Nine microliter of master-mix was filled in the glass capillaries and 1 μL volume, containing 25 ng reverse transcribed total RNA, was added as PCR template. Capillaries were closed, centrifuged, and placed into the rotor. To improve SYBR Green I quantification, fluorescence acquisition was performed at an elevated product specific temperature level (40). It melts the unspecific PCR products at the elevated temperature, e.g., primer dimers, eliminates the non-specific fluorescence signal and ensures an accurate quantification of the desired product. Temperatures for the elevated fluorescence acquisition in the fourth segment are listed in Table 2.

Prior to amplification an initial denaturation step (10 min at 95°C) ensured complete denaturation of the cDNA. Product specific PCR cycle conditions for all receptor subtypes and the housekeeping genes are summarized in Table 2. After the last amplification cycle, PCR products were specified in a melting curve analysis to ensure that they were specific amplification products.

To verify the specificity of each PCR quantification method the PCR amplificates were sequenced. For each product, a PCR run with a 25 μL reaction volume was performed to gain sufficient amounts of PCR amplificates. DNA (5 μL) was applied on a 4% agarose gel

Table 2. Product specific LightCycler PCR conditions for amplification and high temperature fluorescence acquisition (40) melting temperature of bovine 5-HTR subtype PCR products.

	Denaturation		Primer annealing		Elongation		Fluorescence acquisition		Melting temperature
Product	(°C)	(sec)	(°C)	(sec)	(°C)	(sec)	(°C)	(sec)	(°C)
5-HTR_{1A}	95	15	60	10	72	21	85	3	91
5-HTR_{1B}	95	15	61	10	72	24	83	3	90
5-HTR_{1D}	95	15	60	10	72	28	78	3	89
5-HTR_{1F}	95	15	60	10	72	23	80	3	85
5-HTR_{2A}	95	15	60	10	72	25	72	—	88
5-HTR_{2B}	95	15	59	10	72	30	78	3	85
5-HTR_{2C}	95	15	59	10	72	23	81	3	84
5-HTR_4	95	15	60	10	72	21	78	3	87
GAPDH[a]	95	15	60	10	72	20	72	—	88
UbC[b]	95	15	60	10	72	30	72	—	88
18S[c]	95	15	60	10	72	30	72	—	88

[a]Glyceraldehyde-phosphate-dehydrogenase.
[b]Ubiquitin.
[c]18S ribosomal RNA gene.

to check for the presence of single bands and to confirm that there was enough DNA for sequencing. The remaining 20 μL of PCR products were purified with the High Pure PCR purification kit (Roche Molecular Diagnostics, Rotkreuz, Switzerland) and directly sequenced with the Rhodamine dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) using the appropriate PCR primers. After purification of sequencing products by ethanol precipitation, they were run on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) at the Institute of Veterinary Bacteriology, Faculty of Veterinary Medicine, University of Berne, Switzerland. Sequences were edited and proof-read in both directions using Sequencher™ (GeneCodes, Ann Arbor, MI). On agarose gel electrophoresis all PCR products moved with one single band and showed the expected size. Furthermore, melting temperatures that were performed by the LightCycler in a melting curve analysis program after the last amplification cycle demonstrated specific PCR products.

Ubiquitin (UbC), glyceraldehyde-phosphate-dehydrogenase (GAPDH), and 18S ribosomal RNA gene (18S) were chosen to confirm constant housekeeping gene expression levels in the investigated cDNA samples. Mean values ± SD of crossing point (CP) values of brain, abomasum, and intestine pools for UbC, GAPDH, and 18S are shown in Table 3. Spearman correlations between the CPs of the 3 different housekeeping genes calculated for each tissue pool are shown in Table 4.

Mathematical Evaluations and Statistical Analyses

Expression of mRNA was evaluated by amplification curve analysis of the LightCycler real-time RT-PCR. SYBR Green I (DNA binding dye) incorporated into double stranded DNA (dsDNA) emits fluorescence of increasing intensity with cycle number, reflecting target amplification. The exponential growth phase of the PCR begins when the fluorescence signal from accumulated PCR product is greater than the background fluorescence. To eliminate non-informative fluorescence background points, a fluorescence threshold is set to the exponential portion of the amplification curve as low as possible without including any background points. The intersection of the threshold line and the amplification curve represents the CP value (41). Crossing points for each receptor subtype were determined using the second derivate maximum method (41).

Table 3. Means ± SD of housekeeping genes in CPs.

	GAPDH[a] (CP)	UbC[b] (CP)	18S[c] (CP)
CNS[d]	20.55 ± 0.70	20.61 ± 0.57	15.71 ± 0.63
Abomasum[e]	19.61 ± 0.27	19.71 ± 0.30	15.04 ± 0.38
Intestine[f]	20.40 ± 0.26	19.18 ± 0.29	15.83 ± 0.71

[a]Glyceraldehyde phosphate dehydrogenase.
[b]Ubiquitin.
[c]18S ribosomal RNA gene.
[d]Central nervous system: thalamus, hypothalamus, cortex (lobus piriformis).
[e]Abomasum: fundus, corpus, antrum.
[f]Intestine: ileum, caecum, PLAC, spiral colon.

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Table 4. Correlations among housekeeping genes (expression ratio or CP) within tissues.

	GAPDH[d]	UBQ[e]	18S[f]
CNS[a]			
GAPDH	1	0.67*	0.36
UBQ		1	0.79*
18S			1
Abomasum[b]			
GAPDH	1	0.65*	0.44
UBQ		1	0.46
18S			1
Intestine[c]			
GAPDH	1	0.70*	0.54
UBQ		1	0.57
18S			1

*$P \leq 0.05$.
[a]Central nervous system: thalamus, hypothalamus, cortex (lobus piriformis).
[b]Abomasum: fundus, corpus, antrum.
[c]Intestine: ileum, caecum, PLAC, spiral colon.
[d]Glyceraldehyde phosphate dehydrogenase.
[e]Ubiquitin.
[f]18S ribosomal RNA gene.

356 The amount of target gene cDNA was determined using a relative quantification
357 method, i.e., housekeeping gene expression of each sample was used for normalization of
358 5-HTR expression. The low SD-values of housekeeping gene CP (Table 3) revealed that
359 variation of housekeeping gene expression was generally small and indicated that
360 housekeeping genes were barely regulated. Moreover, in every tissue pool, GAPDH
361 and UbC expression were highly and significantly correlated with each other. Based on
362 that, these two housekeeping genes were considered to be most eligible for normalization
363 of target gene expression. Because GAPDH was used for tissue mapping of human
364 5-HTR mRNA expression (36), we decided to use GAPDH for normalization in this
365 study also.
366 According to Medhurst et al. (36) and Inderwies et al. (38), an optimum efficiency (e)
367 of PCR ($e = 2$) was assumed for calculation of mRNA expression, where the PCR product
368 is duplicated in every cycle. The 5-HTR mRNA expression was indicated in percentage of
369 GAPDH mRNA expression and was calculated as
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$$\frac{1}{2^{(CP[5HTR]-CP[GAPDH])}} \times 100\,\%$$

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374 For statistical analyses the S-PLUS 6.0 professional program package was used (MathSoft
375 Inc., Seattle, WA). Data are presented as means $\pm$ SD and the level of significance was set
376 at $P \leq 0.05$. Descriptive analysis revealed that values of 5-HTR expression normalized to

GAPDH were not derived from a normally distributed population. Therefore, 5-HTR expression traits were logarithmically transformed in order to fulfill assumptions of normality. Differences of receptor expression among tissue pools (brain, abomasum, intestine) within receptor subtype and differences of receptor expression among receptor subtypes within tissue pools were localized using two-way analysis of variance (ANOVA). Follow-up tests (paired *t*-tests) were used for pairwise comparison of 5-HTR mRNA expression within receptor subtypes as well as within tissue pools. Holm corrections (42) were used for adjustment to repeated testing.

RESULTS

Primer and PCR-Product Specificity

Specificity of the desired products in bovine tissue total RNA was documented with melting curve analysis and, additionally, with high resolution gel electrophoresis. Derived mean melting temperatures of PCR products were product-specific and are listed in Table 2.

Comparison between the obtained bovine partial CDSs and the corresponding published human, mouse, and rat complete CDSs was done by CDS alignment (HUSAR® software) at DKFZ (http://genome.dkfzheidelberg.de/biounit/). Results of CDS alignment are shown in Table 5. The percentage of identity between the partial bovine CDSs and the complete CDSs used for primer design was highest for human and lowest for rat complete CDS.

The partial bovine CDSs were translated into amino acid sequences and compared with the corresponding published human, mouse, and rat amino acid sequences using amino acid alignment (HUSAR® software) at DKFZ (http://genome.dkfzheidelberg.de/biounit/). Results of amino acid alignment are shown in Table 6. The percentage of identity between the partial bovine amino acid sequnce and the complete amino acid sequences was highest for human and mouse and lowest for rat complete CDS.

Intra- and inter-assay coefficients of variation (CV) were calculated for each 5-HTR subtype to determine the accuracy of the method. For calculation of the intra-assay CV a pooled cDNA sample was analyzed 10 times in the same LightCycler RT-PCR run and for calculation of inter-assay CV the same pooled cDNA sample was analyzed in 10 different LigthCycler RT-PCR runs. Intra- and inter-assay CVs are shown in Table 7.

Bovine 5-HTR Sequences

All eight 5-HTR subtypes were previously not described as bovine nucleotide sequences. The partial CDS of the eight bovine 5-HTR subtypes described in this study can be downloaded at (http://www.ncbi.nlm.nih.gov/Entrez/index.html). The accession numbers are: **5-HTR$_{1A}$:** *AJ491858*, **5-HTR$_{1B}$:** *AJ491859*, **5-HTR$_{1D}$:** *AJ491860*, **5-HTR$_{1F}$:** *AJ491862*, **5-HTR$_{2A}$:** *AJ491863*, **5-HTR$_{2B}$:** *AJ491864*, **5-HTR$_{2C}$:** *AJ491865*, **5-HTR$_{4}$:** *AJ491866.* The nucleotide sequences as well as the amino acid sequences were highly homologous to the known human, rat, and mouse sequences (Tables 5 and 6). Coding sequence alignment of the bovine partial CDS to human, rat, and mouse complete sequences

Table 5. Identity (%) of bovine 5-HTR partial CDS to human, murine, and rat 5-HTR complete CDS.

Receptor	Bovine partial CDS	Human complete CDS		Murine complete CDS		Rat complete CDS	
	NCBI ac. no.[a]	Identity	NCBI ac. no.[a]	Identity	NCBI ac. no.[a]	Identity	NCBI ac. no.[a]
5-HTR_{1A}	AJ491858	91.5	XM_003692	88.2	NM_008308	86.7	NM_012585
5-HTR_{1B}	AJ491859	94.2	XM_004117	91.1	M85151	90.7	NM_022225
5-HTR_{1D}	AJ491860	89.1	NM_000864	86.9	NM_008309	86.9	NM_012852
5-HTR_{1F}	AJ491862	93.5	NM_000866	86.7	NM_008310	—	—
5-HTR_{2A}	AJ491863	92.9	NM_000621	—	—	89.8	NM_017254
5-HTR_{2B}	AJ491864	91.0	XM_048724	84.7	AJ012488	84.4	NM_017250
5-HTR_{2C}	AJ491865	96.0	NM_000868	93.6	NM_008312	91.1	NM_012765
5-HTR_4	AJ491866	93.2	XM_052171	92.7	NM_008313	91.4	NM_012853

[a]National Center for Biotechnology Information acession number. Web address: http://www.ncbi.nlm.nih.gov/entrez/index.html.

Table 6. Identity (%) of bovine 5-HTR partial amino acid sequences to complete human, murine, and rat 5-HTR amino acid sequences.

Receptor	Bovine partial CDS	Human complete CDS		Murine complete CDS		Rat complete CDS	
	NCBI ac. no.[a]	Identity	NCBI ac. no.[a]	Identity	NCBI ac. no.[a]	Identity	NCBI ac. no.[a]
5-HTR_{1A}	AJ491858	97.1	XM_003692	97.1	NM_008308	97.1	NM_012585
5-HTR_{1B}	AJ491859	97.6	XM_004117	97.6	M85151	97.6	NM_022225
5-HTR_{1D}	AJ491860	88.2	NM_000864	87.9	NM_008309	88.8	NM_012852
5-HTR_{1F}	AJ491862	98.8	NM_000866	93.9	NM_008310	—	—
5-HTR_{2A}	AJ491863	99.1	NM_000621	—	—	98.1	NM_017254
5-HTR_{2B}	AJ491864	91.2	XM_048724	80.3	AJ012488	77.4	NM_017250
5-HTR_{2C}	AJ491865	96.4	NM_000868	100.0	NM_008312	96.4	NM_012765
5-HTR_4	AJ491866	91.8	XM_052171	93.2	NM_008313	89.0	NM_012853

[a]National Center for Biotechnology Information acession number. Web address: http://www.ncbi.nlm.nih.gov/entrez/index.html.

Table 7. Coefficients of variation of real-time RT-PCR methods for bovine 5-HTR mRNA determination.

Receptor	Intra-assay CV (%)	Inter-assay CV (%)
5-HTR_{1A}	0.54	0.64
5-HTR_{1B}	0.52	1.55
5-HTR_{1D}	1.83	0.84
5-HTR_{1F}	1.07	0.98
5-HTR_{2A}	0.86	1.19
5-HTR_{2B}	2.46	1.03
5-HTR_{2C}	0.51	0.58
5-HTR_4	0.66	0.49

revealed that the bovine sequences matched the human, mouse, and rat sequences exactly in the regions where the primer have been designed.

Distribution of mRNA Expression

Expression levels of the various receptor subtypes are shown in Figs. 1 and 2. There was a high variation of 5-HTR subtype mRNA expression within tissue among receptor subtypes, as well as within receptor subtypes among tissues. Except for 5-HTR_{1A} in abomasum and intestine and 5-HTR_{2C} in abomasum, every established bovine 5-HTR subtype could be detected in every tissue pool.

DISCUSSION

In this study, assays for quantitative analysis of bovine 5-HTR mRNA expression were developed and described for the first time. Primer design and optimization was done in the high homology regions of the multiple alignments of published human, mouse, and rat sequences and quantification was done relative to the housekeeping gene GAPDH using the LightCycler SYBR Green I technology. Methods for the determination of eight (5-HTR_{1A}, 5-HTR_{1B}, 5-HTR_{1D}, 5-HTR_{1F}, 5-HTR_{2A}, 5-HTR_{2B}, 5-HTR_{2C}, and 5-HTR_4) of 14 known 5-HT R subtypes could be developed in the bovine species.

The specificities of the amplified nucleotide products were confirmed by agarose gel electrophoresis of the product yielding single bands, melting curve analysis, and sequencing of the product.

The eight new bovine partial CDS were compared with the known human, mouse, and rat complete CDS. Because partial CDS were compared with complete CDS, this comparison of CDS across species might have been biased. Therefore, these results have to be carefully interpreted. Nevertheless, high homologies between the bovine and the human, mouse, and rat sequences were to be expected, because 5-HTR have been highly conserved throughout evolution, as evidenced by molecular cloning (5). Typically, the CDS identity was higher between bovine and human than between bovine and mouse or rat. Due to triplet code redundancy, the homologies of amino acid sequences between

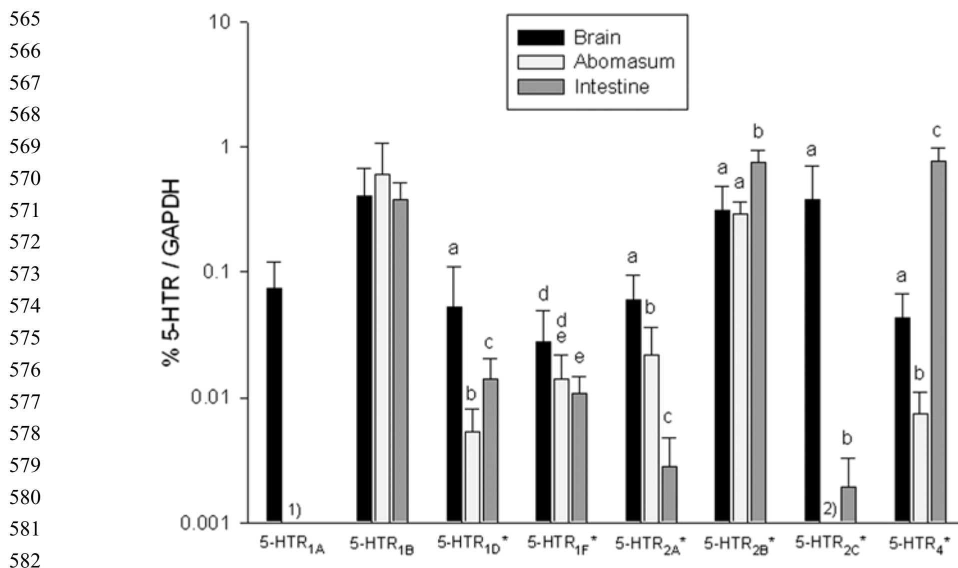

Figure 1. The 5-HTR mRNA expression level in bovine tissues relative to GAPDH mRNA expression grouped by receptor subtype. Values are means ± SD. *Key:* *5-HTR mRNA expression is significantly different within receptor subtype ($P < 0.05$); a–c: significant differences ($P < 0.05$) between two tissues within receptor; d and e: significant differences ($P < 0.1$) between two tissues within receptor; (1) 5-HTR$_{1A}$ mRNA not detectable in abomasum and intestine. (2) 5-HTR$_{2C}$ mRNA not detectable in abomasum.

bovine and human, mouse, and rat were generally even higher than the homologies between nucleotide sequences.

Validation of the eight newly developed assays for quantitative mRNA analysis of bovine 5-HTRs by calculation of intra- and inter-assay CVs revealed that the repeatability of the measurements and the accuracy of the assays were high.

Analysis of housekeeping gene CPs of each sample revealed that GAPDH and UbC were most suitable for normalization of 5-HTR expression. Because GAPDH was used for tissue mapping of human 5-HTR mRNA expression (36), we decided to use GAPDH for normalization in this study also.

The CPs of bovine 5-HTR mRNA analysis were rather high, i.e., expression levels of bovine 5-HTR were rather low and accounted for 0.001% to 1% of GAPDH expression only. However, considering that signaling by serotonin binding to 5-HTR is strongly amplified by second messengers, this fact is not surprising (8).

Expression levels of bovine 5-HTR were comparable to expression levels of human 5-HTR. Thus, expression levels for 5-HTR$_4$ were 0.05% and 0.7% 5-HTR$_4$/GAPDH in human whole brain and small intestine (36) and 0.04% and 0.8% 5-HTR$_4$/GAPDH in bovine brain and intestine pool, respectively.

Interestingly, 5-HTR expression varied considerably among tissues within receptor subtype as well as among receptor subtypes within tissues. Moreover, the tissue with highest 5-HTR mRNA expression levels varied from subtype to subtype, suggesting different effects of 5-HT stimulations within tissues.

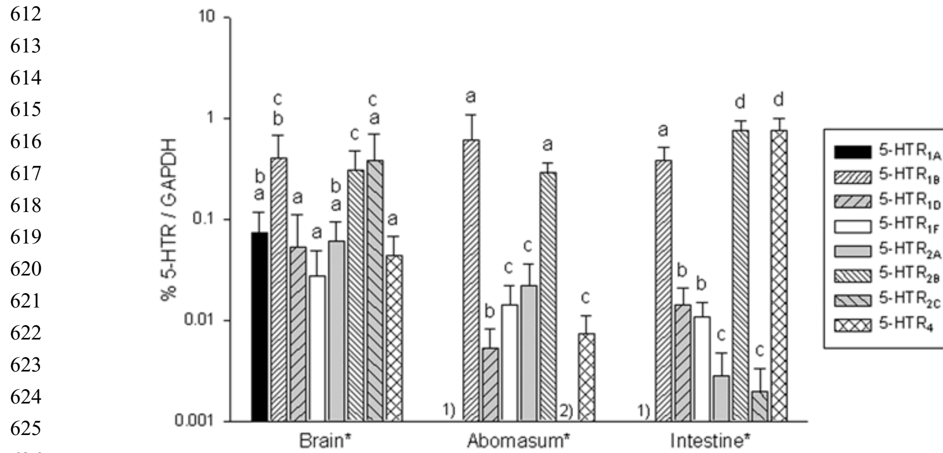

Figure 2. The 5-HTR mRNA expression level in bovine tissues relative to GAPDH mRNA expression grouped by tissue. Values are means ± SD. *Key:* *5-HTR mRNA expression is siginificantly different within tissue ($P < 0.001$); a–d: significant differences ($P < 0.05$) between two receptors within tissue R. (1) 5-HTR$_{1A}$ mRNA not detectable in abomasum and intestine. (2) 5-HTR$_{2C}$ mRNA not detectable in abomasum.

In conclusion, this study demonstrates development and validation of assays for quantitative mRNA analysis of eight bovine 5-HTR subtypes. A first physiological application shows a variable and an obviously tissue-specific distribution of these subtypes in bovine brain, abomasum, and intestine tissue pools. Because the bovine 5-HTR nucleotide sequences were completely unknown before, primers used for bovine cDNA amplification were either derived from human or mouse complete CDS in highly homologous regions across species (human, mouse, and rat). In future, the real-time RT-PCR assays developed in this study will allow for detailed mapping of the bovine tissues with respect to 5-HTR subtype expression. Furthermore, the determination of 5-HTR subtype expression represents an additional tool in research on bovine production diseases such as disorders of GI motility (displaced abomasum, cecal dilatation/displacement) and uterus motility in cattle and completes existing techniques such as measurement of muscular contractions upon specific 5-HTR stimulation in vitro or electromyographical recordings in vivo. However, we have to keep in mind that mRNA determination is not a physiological measure, especially if it is based on partial sequences.

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