The potential role of myocardial serotonin receptor 2B expression in canine dilated cardiomyopathy

Fonfara, S; Hetzel, U; Oyama, M A; Kipar, A

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Best regards
Sonja
Original Article

The potential role of myocardial serotonin 2B receptor expression in canine dilated cardiomyopathy

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Abstract

Serotonin signalling in the heart is mediated by receptor subtype 2B (5-HTR2B). A contribution of serotonin to valvular disease is reported, but myocardial expression of 5-HTR2B and its role in canine dilated cardiomyopathy (DCM) is not known. The aim of this study was to investigate myocardial 5-HTR2B mRNA expression in dogs with DCM and to correlate results with expression of markers for inflammation and remodelling. Myocardial samples from eight healthy dogs, four dogs with DCM, five dogs with cardiac diseases other than DCM and six dogs with systemic non-cardiac diseases were investigated for 5-HTR2B mRNA expression using quantitative PCR (qPCR). The results were compared to mRNA expression of selected cytokines, matrix metalloproteinases (MMP) and tissue inhibitors of matrix metalloproteinase (TIMP). Laser microdissection with subsequent qPCR and immunohistochemistry were employed to identify the cells expressing 5-HTR2B. The myocardium of control dogs showed constitutive 5-HTR2B mRNA expression. In dogs with DCM, 5-HTR2B mRNA values were significantly greater than in all other groups, with highest levels of expression in the left ventricle and right atrium. Myocytes were identified as the source of 5-HTR2B mRNA and protein. A significant positive correlation of 5-HTR2B with expression of several cytokines, MMPs and TIMPs was observed. These findings suggest that serotonin might play a role in normal cardiac structure and function and could contribute to myocardial remodelling and functional impairment in dogs with DCM.

Keywords: Serotonin, Heart failure, Cardiac disease, Dilated cardiomyopathy, Myocardial remodelling
Introduction

Serotonin (5-hydroxytryptamine; 5-HT) is a monoamine neurotransmitter, which is produced from tryptophan by two different tryptophan hydroxylases (TPH), namely, TPH1 and TPH2, found in enterochromaffine cells of the gastrointestinal tract and in neurons of the central nervous system, respectively (Jonnakuty and Gragnoli, 2008). Serotonin is involved in platelet function, vascular and non-vascular smooth muscle contraction as well as cardiac function (Nebigil and Maroteaux, 2003; Jonnakuty and Gragnoli, 2008). It is rapidly removed from the circulation, via cellular uptake by the serotonin transporter (SERT) and subsequently stored in platelets or metabolised in pulmonary vascular endothelial cells and hepatocytes by monoamine oxidase (Jonnakuty and Gragnoli, 2008). However, high circulating 5-HT concentrations or administration of serotonergic drugs are associated with arrhythmia and valvulopathy (Sheline et al., 1997; reviewed by Kaumann and Levy, 2006; Elangbam et al., 2008; Orton et al., 2012). Furthermore, 5-HT is suspected to be involved in myxomatous valvular disease (MVD) in humans and dogs (Fitzgerald et al., 2000; Arndt et al., 2009; Oyama and Levy, 2010).

Serotonin exerts its effect through seven different receptor groups (5-HTR1 to 5-HTR7), composed of several receptor subtypes (Elangbam et al., 2005; Kaumann and Levy, 2006), all of which are members of the G-protein-coupled receptor superfamily. In the cardiovascular system, the receptor subtypes 5-HTR1B, 2A, 2B, 4 and 7 can be found, of which the receptor subtype 5-HTR2B, which is present on endothelial cells, smooth muscle cells, fibroblasts, valvular interstitial cells and cardiomyocytes, is suspected to be involved in cardiac remodelling and development of valvulopathies (Rothman et al., 2000; Kaumann and Levy, 2006; Disatian and Orton, 2009; Oyama and Levy, 2010; Hutcheson et al., 2011).
Activation of 5-HTR2B has been found to stimulate phospholipase C and A2, both of which increase the intracellular calcium concentration. In addition, downstream signalling, involving extracellular-signal regulated kinase (ERK), mediates proliferative effects by inducing transcription of transforming growth factor (TGF)-β and other effector genes, such as matrix metalloproteinases (MMP) and bone morphogenic protein, which potentially contribute to the pathogenesis of mitral valve disease (Disatian and Orton, 2009; Lacerda et al., 2012a; Orton et al., 2012). Furthermore, activation of 5-HTR2B has been shown to increase expression of interleukin (IL)-1, IL-6 and tumor necrosis factor alpha (TNF-α) mRNA and protein in murine cardiac fibroblasts (Jaffre et al., 2004). These inflammatory cytokines are known to be elevated in the blood of humans and dogs affected with congestive heart failure (CHF) and are involved in cardiac inflammation and remodelling (Anker and von Haehling, 2004; Fonfara et al., 2011). Tensile strain has been suspected to increase TPH and 5-HTR2B and reduce SERT in canine septal mitral valve leaflets, which might result in local 5-HT synthesis and prolonged 5-HT activity (Elangbam et al., 2008; Scruggs et al., 2010; Lacerda et al., 2012a; Lacerda et al., 2012b).

The role of 5-HT and its receptors in canine DCM is not fully understood. Dilated cardiomyopathy is associated with left ventricular dilatation and ventricular wall thinning, therefore increasing wall stress. Increased ventricular wall stress is likely to stimulate mechanoreceptors, which could cause local 5-HT production and increased 5-HTR2B expression, as reported for valve leaflets exposed to tensile strain (Disatian and Orton, 2009; Lacerda et al., 2012b). This appears likely, since in rats, chronic 5-HT administration has been shown to increase valvular 5-HTR2B transcription (Elangbam et al., 2008). In humans with heart failure, 5-HT plasma concentrations are also elevated, which has led to the assumption that heart failure is mediated by 5-HTR2B (Jaffre et al., 2009; Shyu, 2009).
It is suspected that through induction of IL-1, IL-6, TNF-α and TGF-β1, 5-HT contributes to cardiomyocyte hypertrophy, increased fibrosis and ventricular stiffness leading to reduced cardiac contractility and heart disease (Anker and von Haehling, 2004; Jaffre et al., 2004; Disatian and Orton, 2009; Jaffre et al., 2009). Increased concentrations of TGF-β1, a potent profibrotic cytokine, have been reported in canine MVD (Aupperle et al., 2008; Disatian and Orton, 2009) and CHF (Fonfara et al., 2011). We have recently shown that dogs with end-stage cardiac diseases exhibit increased myocardial mRNA expression of inflammatory cytokines, TGF-β1, MMP-2 as well as tissue inhibitor of matrix metalloproteinase (TIMP)-1 and TIMP-2 (Fonfara et al., 2013a; Fonfara et al., 2013b). Considering these results and the characteristic pathological changes in canine DCM, we hypothesize that the 5-HT system plays a role in the pathogenesis of canine DCM. We therefore designed a study to investigate cardiac expression of 5-HTR2B and its association with expression of IL-1, IL-6, TNF-α, TGF-β1, MMP-1, -2, -3, -13, TIMP-1 and -2, comparing healthy control dogs with other groups of dogs affected with DCM, other cardiac diseases or systemic non-cardiac disease.

**Material and methods**

*Animals and tissues*

Details of clinical cases are shown in Table 1 and consist of dogs affected with DCM (group 2; n = 4), cardiac diseases other than DCM (group 3; n = 5) and dogs with systemic, non-cardiac disease (group 4; n = 6) (Fonfara et al., 2013a; Fonfara et al., 2013b). Eight control Beagles (group 1a) were used for the quantitative PCR assay (four each entire male and female, sourced from a pharmaceutical company with a median age of 2.75 years). The dogs were euthanized and post-mortem examination performed on site. Samples from the
myocardium (left and right atrium [LA, RA], left and right ventricle [LV, RV]) were immersed in RNA later (Ambion) and provided for this project. Two Doberman Pinscher dogs (one female neutered 2 years old, one male entire 6 years old), with no gross or histological evidence of cardiac disease, served as controls for the immunohistological examination.

All of the dogs with cardiac and systemic diseases were clinical cases that had undergone diagnostic investigations according to their underlying disease and presenting clinical signs. Investigations were performed by clinical specialists in their respective fields or board-registered residents under supervision. For cardiac cases, investigations included a cardiac work-up, comprising blood pressure measurement, electrocardiography, echocardiography and thoracic radiography, at different time points prior to death (Fonfara et al., 2013b). Diagnoses were made, based on applying standardised criteria for clinical assessment of cardiac cases. For classification of heart failure, the ABCD scheme was used (Strickland, 2008). Dogs had been euthanized upon owners’ request, due to poor prognosis and impaired quality of life, with one exception, where a dog developed ventricular fibrillation and died (Fonfara et al., 2013b). Informed consent was obtained from owners prior to inclusion into the study and samples were anonymised by assigning identification numbers. The study was approved by the University of Bristol Committee on Research Ethics.

Hearts were removed within 1 h of death and examined for any gross pathological abnormalities. Myocardial samples (interventricular septum [IVS], RA, RV, LA, LV) were collected and stored in RNA later at -20 °C until use. Hearts were subsequently fixed in 10% formalin for a minimum of 48 h and tissue samples from the same sites as those for RNA
extraction were prepared and paraffin wax embedded, according to routine procedures for histological and immunohistological examinations. The hearts from the immunohistology control dogs (group 1b) were formalin-fixed and subsequently processed as for the other hearts.

Laser microdissection

A left ventricular sample from a dog with DCM stored in RNAlater was embedded in OCT compound (Tissue-Tek) and frozen at -40 °C. Cryosections (8-10 μm) were prepared and placed onto specific membrane slides (Carl Zeiss), which had been incubated in dry heat at 180 °C for 4 h prior to use, in order to inactivate RNase enzymes. The slides with cryosections were air dried, stained with haematoxylin as recommended by the manufacturer (Carl Zeiss) and immediately used.

Cardiomyocytes were isolated with a Zeiss Palm microbeam laser microdissection microscope, using a 40× lens. Cutting was performed with 52-55% energy, 60-62% focus and 10% speed and collection with 65% energy and 47% focus. Three samples of approximately 100-150 cardiomyocytes were collected into a 500 μL adhesive cap tube (Carl Zeiss). After collection, tissue was lysed into 50 μL RLT buffer containing 2-mercaptoethanol (Qiagen). Samples were subsequently frozen at -20 °C for 18-22 h and subsequently RNA isolation was performed using the RNeasy Minikit (Qiagen).

Quantitative assessment of 5-HTR2B mRNA expression

Total RNA was extracted from myocardial samples and cDNA synthesized as reported previously (Fonfara et al., 2011b). For the samples obtained by laser microdissection, 14 μL RNase-free water was used to elute RNA from the column prior to cDNA synthesis.
Published primer sequences were used for the canine housekeeping gene GAPDH: 5’CTGGGGCTCACTTGAAAGG3’ and 5’CAAACATGGGGCATCAG3’, respectively, and for 5-HTR2B 5’CCCAATGAGGCTCTGCAGTT3’ and 5’CTGTGATGAGAAGTGTATCTAGTAGAATGATT3’, respectively, and 5-HTR2B

[Editor comment: might be better to put primer sequences here. You reference three publications for two primer pairs] (Oyama and Chittur, 2006; Fonfara et al., 2013a; Fonfara et al., 2013b). The primer efficiency of the GAPDH primer pair was 100% and of 5-HTR2B 97%. PCR was performed and analyzed according to standard protocols, the expression of 5-HTR2B was normalized to GAPDH expression (relative expression) and calculated via the 2^△△Ct method, as previously reported (Fonfara et al., 2013a; Fonfara et al., 2013b).

Statistical analysis and correlation of 5-HTR2B mRNA with cytokine, MMP and TIMP expression

For the statistical analysis of the quantitative PCR results, Minitab 16 was used. Following performance of basic descriptive statistics, 5-HTR2B mRNA values were log transformed to improve normality and the model assumptions necessary for parametric analysis. For comparison of different groups and/or cardiac regions, one-way ANOVA tests were employed. Results are displayed as mean and standard deviation.

To test for a potential correlation between 5-HTR2B and cytokine, MMP or TIMP expression in the hearts of dogs with DCM, the qPCR results for IL-1, IL-6, TNF-α, TGF-β1, MMP-1, 2, -3, -13, TIMP-1 and TIMP-2, obtained from the same myocardial samples, were used (Fonfara et al., 2013a; Fonfara et al., 2013b). Expression was examined with scatterplots
and then tested with the Pearson correlation test. Statistical significance was defined as $P < 0.05$.

**Histology and immunohistochemistry**

For the histological examination, 3-5 μm thick sections were prepared from the paraffin wax-embedded tissue blocks and stained with haematoxylin and eosin (HE). Immunohistochemistry for 5-HTR2B was performed on sections from the RA and the RV and/or LV using a murine anti-human 5-HTR2B antibody (clone A72-1; Beckton Dickinson) and the horseradish peroxidase method (HRP mouse kit, Dako), following antigen retrieval by incubation in citrate buffer (pH 6.0) and microwaving (Disatian and Orton, 2009). Consecutive sections were incubated with a isotype-control mouse monoclonal antibody and sections from canine stomach, tissue from a case of suppurative myocarditis and smooth muscle cells present in myocardial arteries served as positive controls.

**Results**

**Pathology and histopathology**

The hearts of dogs with systemic diseases and clinical diagnoses of cardiac diseases other than DCM did not exhibit any gross or histological changes, i.e. there was no evidence of degenerative, neoplastic or inflammatory changes (Fonfara et al., 2013b). The hearts of dogs with DCM showed the typical gross changes (cardiomegaly, biventricular dilatation, myocardial eccentric hypertrophy). Histologically, interstitial, subendo- and subepicardial fibrosis, lipomatosi cordis, leukocyte infiltration, and focal cardiomyocyte necrosis was observed, as previously reported (Fonfara et al., 2013b).

**Canine cardiac 5-HTR2B expression**
Expression of 5-HTR2B mRNA was detected in the myocardium of control dogs in all examined locations (RA, RV, LA, LV), with some individual variation in quantitative values (Fig. 1). In the other groups, 5-HTR2B expression was also consistently observed, including in the IVS, which was not available for examination in control dogs. There was a greater individual variation in the disease groups (Table 2), although for groups 3 and 4, 5-HTR2B values did not differ significantly from those of control dogs. In contrast, dogs with DCM (group 2) demonstrated significantly greater 5-HTR2B mRNA expression when compared to all other groups (Table 2).

The results of the RT-PCR performed on isolated cardiomyocytes of the LV of a dog affected with DCM, showed that the cardiomyocytes were expressing 5-HTR2B mRNA and the immunohistochemical staining of both control and diseased dogs confirmed that cardiomyocytes also synthesised 5-HTR2B protein (Fig. 2). Immunohistochemistry showed 5-HTR2B protein expression not only in cardiomyocytes, but also in vascular smooth muscle cells and infiltrating as well as intravascular neutrophils (Fig. 2). Immunostaining in cardiomyocytes was generally weak and often patchy or with negative fibers intermingled with positive fibers. However, staining in cardiomyocytes was generally most intense in the right atrium, a finding that was supported by the general trend for greatest mRNA expression at this location (Fig. 1).

Disease-associated differences in 5-HTR2B mRNA expression values, concentrations and patterns

A comparison of 5-HTR2B mRNA values, concentrations in the different groups showed comparable expression levels in controls dogs and those with cardiac diseases other than DCM, or dogs with systemic diseases ($P = 0.46$). However, in dogs with DCM, mRNA
values concentrations were significantly greater than in all other groups ($P = 0.002$; Fig. 1).

This difference was primarily due to greater 5-HTR2B mRNA values-concentrations in the LV ($P = 0.005$) and the RA ($P = 0.027$) of dogs with DCM, when the different cardiac regions were compared between groups. In dogs with DCM, the same significant difference in 5-HTR2B mRNA ($P = 0.001$) was observed between the different regions, with 5-HTR2B mRNA concentrations values in the RA also being significantly higher than in the LV ($P = 0.047$; Fig. 1). None of the other groups exhibited significant differences in mRNA concentrations values comparing different cardiac locations.

The immunohistochemical results confirmed the mRNA expression data, since staining of cardiomyocytes for 5-HTR2B was more consistent and more intense in the RA, compared to other locations in the DCM dogs, and compared to the same location in dogs from other groups (Fig. 2B, C).

Correlation between 5-HTR2B and cytokine, MMP and TIMP expression in the myocardium of dogs with DCM

We have previously shown upregulation of cytokine, MMP and TIMP mRNA expression in the myocardium of dogs with DCM (Fonfara et al., 2013a; Fonfara et al., 2013b). Using the qPCR data generated from the same RNA extracts and applying the Pearson’s correlation test, a significant positive correlation was seen between 5-HTR2B mRNA expression and IL-1 ($r = 0.719$, $P < 0.001$; Fig. 3a), IL-6 ($r = 0.624$, $P = 0.003$), TNF-α ($r = 0.661$, $P = 0.002$), TGF-β1 ($r = 0.848$, $P < 0.001$; Fig. 3b), MMP-2 ($r = 0.493$, $P = 0.027$), MMP-13 ($r = 0.770$, $P < 0.001$; Fig. 3a), TIMP-1 ($r = 0.729$, $P < 0.001$; Fig. 3b) and TIMP-2 ($r = 0.826$, $P < 0.001$). [Editor comment: should you add an additional figure to illustrate this data? What are the $r$ values?]
Discussion

The present study assesses the constitutive expression of 5-HTR2B in the myocardium of dogs and the changes in expression pattern and intensity in association with canine DCM. The examination of control dogs showed that the myocardium constitutively expresses 5-HTR2B, suggesting a role for 5-HT and its cardiac receptor in normal cardiac structure and function (Nebigil et al., 2001; Nebigil et al., 2003; Liang et al., 2006). There were no significant differences in regional expression of 5-HTR2B mRNA, but a trend for higher expression in the RA was observed.

Our results indicate that severe systemic diseases and non-DCM cardiac diseases do not influence 5-HTR2B expression, whereas canine DCM is associated with a significant increase in the RA and, to a lesser, but still significant extent, the LV. This 5-HTR2B upregulation correlated with increased IL-1, IL-6, TNF-α, TGF-β1, MMP-2, MMP-13, TIMP-1 and TIMP-2 mRNA expression. Using laser microdissection and immunohistology, we were able to confirm that cardiomyocytes synthesize 5-HTR2B, which is similar to previously reported results for canine MVD and cardiomyocytes in mice and humans (Choi and Maroteaux, 1996; Kaumann and Levy, 2006; Liang et al., 2006; Disatian and Orton, 2009; Oyama and Levy, 2010; Orton et al., 2012).

Serotonin, in association with increased 5-HTR2B and reduced SERT production, has been reported in dogs with MVD (Oyama and Chittur, 2006; Arndt et al., 2009; Disatian and Orton, 2009; Scruggs et al., 2010). The results of the present study indicate that 5-HTR2B also plays a role in canine DCM. In contrast to the results of the current study, in mouse models of DCM, reduced 5-HTR2B protein expression was observed, whereas increased 5-
HTR2B expression caused cardiac hypertrophy (Nebigil et al., 2001; Nebigil et al., 2003). Since the dogs with DCM in our study exhibited ventricular eccentric hypertrophy and were all in decompensated CHF, these results may not be entirely contradictory, but are on the other hand not fully comparable.

In humans, CHF has been shown to be associated with an increase of 5-HT independently of the type of cardiac disease (Jaffre et al., 2009). Since chronic 5-HT administration results in increased 5-HTR2B expression (Elangbam et al., 2008), it is possible that 5-HT is also chronically elevated in dogs that develop DCM, resulting in increased 5-HTR2B transcription, thereby suggesting that CHF and not the underlying cardiac disease is responsible for the increase of 5-HT and its receptor. However, if high 5-HT concentrations would be the cause of 5-HTR2B upregulation in DCM, it can be expected that all regions investigated and not just the RA and LV would increase 5-HTR2B gene expression. Instead, our results suggested local changes in the 5-HT system, and upregulation of 5-HTR2B in the LV (which is mainly affected in canine DCM) further supports this hypothesis.

Interestingly in humans, administration of a 5-HT re-uptake inhibitor has been reported to cause DCM and cardiogenic shock, but myocardial 5-HTR2B was not investigated in this patient (Charniot et al., 2010). It is also possible that an increase of TPH1 and phosphorylated ERK, or a reduction of SERT would result in enhanced serotonin production and signalling, as reported for valvular diseases (Elangbam et al., 2008; Disatian and Orton, 2009; Scruggs et al., 2010; Lacerda et al., 2012a; Lacerda et al., 2012b). Further investigations, in particular assessment of circulating serotonin concentrations and the myocardial signal transduction pathway, would be needed to confirm this. The lack of evidence of increased 5-HTR2B gene
expression in dogs with cardiac diseases other than DCM might be a consequence of the case selection, i.e. dogs with a variety of cardiac diseases other than DCM were included into this group.

We identified a positive correlation between 5-HTR2B expression and IL-1, IL-6, TNF-α, TGF-β1, MMP-2, MMP-13, TIMP-1 and TIMP-2 mRNA in the myocardium of dogs with DCM. Others have reported that 5-HT can induce IL-1, IL-6, TNF-α, TGF-β1, MMP-1, MMP-3 and MMP-13 (Jaffre et al., 2004; Yabanoglu et al., 2009; Lacerda et al., 2012b). The inflammatory cytokines IL-1, IL-6 and TNF-α are important mediators of myocardial inflammation and affect myocardial contractility (Anker and von Haepling, 2004). The cytokine TGF-β1 mediates growth of cardiomyocytes (Schultz et al., 2002) and is suspected to impair mitochondrial energy metabolism (Huntgeburth et al., 2011) and might therefore be involved in eccentric cardiac hypertrophy and contribute to systolic dysfunction in dogs with DCM and CHF. Furthermore, myofibroblast activation by TGF-β1 alongside mechanical strain is suspected to lead to increased stiffening of heart valves (Orton et al., 2012), and could also be involved in increased ventricular stiffness and functional impairment in dogs with DCM and CHF.

Matrix metalloproteinases and TIMPs are important regulators of cardiac remodeling (Spinale, 2007). It has been reported that 5-HT induces MMP-3 and MMP-13 expression in cardiac fibroblasts (Yabanoglu et al., 2009) and an increased MMP-1 and MMP-13 protein production in mitral valve leaflets exposed to tensile strain was suspected to be caused by 5-HTR2B stimulation (Lacerda et al., 2012a; Lacerda et al., 2012b). In the present study, a significant positive correlation of 5-HTR2B with MMP-2, MMP-13, TIMP-1 and TIMP-2 expression was detected in myocardial samples of dogs with DCM and CHF. Matrix
metalloproteinase 2, a gelatinase, and MMP-13, a collagenase, might be involved in matrix
degradation and ventricular dilatation, whereas TIMP-1 and -2 might contribute to increased
fibrosis and ventricular stiffness in these dogs (Spinale, 2007).

Increased 5-HTR2B mRNA expression in the RA and the further increase in dogs with
DCM is of interest. In humans, elevated 5-HT concentrations have been reported to cause
pathological changes in the right heart valves, whereas the left heart valves are presumed to
be protected by 5-HT breakdown by pulmonary monoamine oxidase (Hutcheson et al., 2011).
Additionally, increased 5-HT concentrations have been shown to produce arrhythmia,
including atrial fibrillation (AF) (Sheline et al., 1997). An increase of 5-HTR2B in the RA
might reflect enhanced 5-HT signalling. This would contribute to the structural remodelling
of the RA, which is an important factor in the development of AF (Brundel et al., 2005).
Also, we recently reported increased expression of IL-1, TNF-α and TGF-β1 in the RA and of
MMP-2 and MMP-13 in both atria of dogs with AF (Fonfara et al., 2013a; Fonfara et al.,
2013b). TGF-β1 is known to modulate atrial ion channels, potential promoting AF (Ramos-
Mondragon et al., 2011) and MMP-2 and -13 might cause atrial dilatation, which is
frequently associated with AF. Therefore, increased 5-HTR2B expression in the RA, together
with upregulation of inflammatory cytokines, TGF-β1, MMP-2 and -13 might contribute to
development of AF, a clinical feature observed in three of the four dogs with DCM.

Arrhythmia might also be caused by 5-HTR2B-induced activation of phospholipase C
and A2, which results in an increase in intracellular calcium concentration and disrupted
calcium handling (Hutcheson et al., 2011). This might be one of the mechanisms underlying
reduced myocardial function observed in dogs with DCM and CHF (Martin et al., 2009;
Oyama et al., 2009).
The study has a number of limitations, including the small number of dogs used, the heterogeneity of groups 3 and 4 and the age difference between the control animals and those in the other groups. Larger sample sizes and groups with more homogeneous phenotypes would have allowed a comparison of different cardiac diseases and might have provided more significant results. Investigation of 5-HTR2B expression in isolation, without studying further components of the signal transduction pathway, limits interpretation of the study findings, in terms of how receptor upregulation might impact on cellular responses. However, a positive correlation of 5-HTR2B expression with increased expression of the downstream gene products of the signal transduction pathway was present. Only dogs with end-stage diseases were included, which also excluded investigation of the involvement of 5-HTR2B in progression of DCM.

Conclusions

Constitutive expression of 5-HTR2B mRNA in myocardial samples of young Beagle dogs suggests an involvement of 5-HT and its receptor in normal cardiac structure and function. Increased 5-HTR2B mRNA expression and a positive correlation with IL-1, IL-6, TNF-α, TGF-β1, MMP-2, MMP-13, TIMP-1 and TIMP-2 in dogs with DCM and CHF suggests a contribution of 5-HT to structural myocardial remodelling and functional impairment in these dogs. Further investigations of the role of 5-HT, its receptor and the signal transduction pathway in the progression of canine DCM are needed in particular to investigate any potential therapeutic implications.

Conflict of interest statement
None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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**Table 1.** Details of dogs in each of the study groups.

<table>
<thead>
<tr>
<th>Study group</th>
<th>Breed</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Disease</th>
<th>CHIEF classification</th>
<th>BW (kg)</th>
<th>AF (Y/N)</th>
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<tbody>
<tr>
<td>2</td>
<td>Doberman</td>
<td>10</td>
<td>NF</td>
<td>Dilated cardiomyopathy</td>
<td>D</td>
<td>34.8</td>
<td>Y</td>
</tr>
<tr>
<td>2</td>
<td>Doberman</td>
<td>8</td>
<td>F</td>
<td>Dilated cardiomyopathy</td>
<td>D</td>
<td>39.6</td>
<td>Y</td>
</tr>
<tr>
<td>2</td>
<td>Bullmastiff</td>
<td>7</td>
<td>M</td>
<td>Dilated cardiomyopathy</td>
<td>D</td>
<td>54</td>
<td>N</td>
</tr>
<tr>
<td>2</td>
<td>Great Dane</td>
<td>6</td>
<td>M</td>
<td>Dilated cardiomyopathy</td>
<td>D</td>
<td>70.8</td>
<td>Y</td>
</tr>
<tr>
<td>3</td>
<td>Boxer</td>
<td>11</td>
<td>M</td>
<td>Aortic stenosis</td>
<td>B</td>
<td>26</td>
<td>N</td>
</tr>
<tr>
<td>3</td>
<td>Labrador</td>
<td>0.2</td>
<td>M</td>
<td>Tricuspid dysplasia</td>
<td>C3</td>
<td>7.8</td>
<td>N</td>
</tr>
<tr>
<td>3</td>
<td>Labrador</td>
<td>9</td>
<td>NF</td>
<td>Pulmonic stenosis</td>
<td>B</td>
<td>32.5</td>
<td>N</td>
</tr>
<tr>
<td>3</td>
<td>Labrador</td>
<td>6</td>
<td>NM</td>
<td>Arrhythmogenic cardiomyopathy</td>
<td>C3</td>
<td>32</td>
<td>N</td>
</tr>
<tr>
<td>3</td>
<td>German Shepherd</td>
<td>14</td>
<td>NF</td>
<td>Myxomatous valvular disease</td>
<td>D</td>
<td>22.7</td>
<td>Y</td>
</tr>
<tr>
<td>4</td>
<td>Boxer</td>
<td>7.5</td>
<td>M</td>
<td>Brain tumour</td>
<td>-</td>
<td>37.5</td>
<td>N</td>
</tr>
<tr>
<td>4</td>
<td>Cocker</td>
<td>8</td>
<td>NF</td>
<td>Brain tumour</td>
<td>-</td>
<td>14.4</td>
<td>N</td>
</tr>
<tr>
<td>4</td>
<td>Cross</td>
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<td>NF</td>
<td>Lymphoma</td>
<td>-</td>
<td>9.6</td>
<td>N</td>
</tr>
<tr>
<td>4</td>
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<td>11</td>
<td>NF</td>
<td>Pancreatic carcinoma</td>
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<td>N</td>
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<td>0.75</td>
<td>M</td>
<td>Secondary hyperparathyroidism</td>
<td>-</td>
<td>24.55</td>
<td>N</td>
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<tr>
<td>4</td>
<td>Labrador</td>
<td>0.5</td>
<td>M</td>
<td>Spinal fracture</td>
<td>-</td>
<td>27.3</td>
<td>N</td>
</tr>
</tbody>
</table>

CHIEF, Canine Heart Failure International Expert Forum; AF, atrial fibrillation; BW, body weight; CHF, congestive heart failure; F, female; M, male; N, no; NF, neutered female; NM, neutered male; Y, yes.
Table 2. Relative 5-HTRB2 mRNA expression in different myocardial regions of control dogs (group 1), dogs with dilated cardiomyopathy (group 2), those with cardiac diseases other than dilated cardiomyopathy (group 3) and those with systemic non-cardiac diseases (group 4). Results are displayed as mean (top value) and standard deviation (bottom value).

<table>
<thead>
<tr>
<th>Region</th>
<th>Group 1 (n = 8)</th>
<th>Group 2 (n = 4)</th>
<th>Group 3 (n = 5)</th>
<th>Group 4 (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interventricular septum</td>
<td>n.d.</td>
<td>1.64 (0.44)¹</td>
<td>1.37 (0.46)</td>
<td>1.34 (0.50)</td>
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<tr>
<td>Left atrium</td>
<td>1.39 (0.37)²</td>
<td>1.62 (0.26)¹</td>
<td>1.40 (0.33)</td>
<td>1.32 (0.49)</td>
</tr>
<tr>
<td>Left ventricle</td>
<td>1.47 (0.20)²</td>
<td>2.12 (0.26)¹²</td>
<td>1.20 (0.36)²</td>
<td>1.22 (0.55)²</td>
</tr>
<tr>
<td>Right atrium</td>
<td>1.97 (0.42)³</td>
<td>2.89 (0.55)¹³</td>
<td>1.52 (0.90)³</td>
<td>2.02 (0.57)³</td>
</tr>
<tr>
<td>Right ventricle</td>
<td>1.27 (0.54)</td>
<td>1.54 (0.34)¹</td>
<td>1.46 (0.46)</td>
<td>1.08 (0.57)</td>
</tr>
<tr>
<td>All samples</td>
<td>1.54 (0.45)⁴</td>
<td>1.96 (0.62)⁴</td>
<td>1.39 (0.60)⁴</td>
<td>1.40 (0.60)⁴</td>
</tr>
</tbody>
</table>

Significant difference between results: ¹P = 0.001, ²P = 0.005, ³P = 0.027, ⁴P = 0.002.

n.d., not determined.

¹ A significant difference in 5-HTRB2 mRNA expression was present comparing cardiac regions of group 2 (P = 0.001).

²,³ 5-HTRB2 mRNA expression was greater in the left ventricle (²P = 0.005) and right atrium (³P = 0.027) of dogs from group 2, comparing these regions between groups.

⁴ A significant difference in 5-HTRB2 mRNA expression was present comparing all samples of the different groups (P = 0.002).

[Editor comment: what does # represent? Can you be more precise about what the statistics represent?]
Figure Legends

**Fig. 1.** Relative 5-HTR2B mRNA expression (normalized to GAPDH expression) in myocardial samples of different cardiac regions [interventricular septum (IVS), left atrium (LA), left ventricle (LV), right atrium (RA) right ventricle (RV)] comparing control dogs (group 1, \( n = 8 \)), dogs with dilated cardiomyopathy (group 2, \( n = 4 \)), dogs with cardiac diseases other than dilated cardiomyopathy (group 3, \( n = 5 \)) and dogs with systemic non-cardiac diseases (group 4, \( n = 6 \)). Significantly greater 5-HTR2B mRNA expression was present in left ventricular and right atrial samples of dogs with DCM (\( P < 0.05 \)). Individual value plot with mean \( \oplus \) and median \( \ominus \). [Editor comment: Is the data normally distributed (I assume not) in which case it might be better just to have a median line. You need a y-axis label.]

**Fig. 2.** Immunohistochemical analysis of 5-HTR2B protein expression in the myocardium of the right atrium. A) Control dog. Cardiomyocytes exhibiting a weak positive cytoplasmic reaction for 5-HTR2B (arrows) are found close to negative cardiomyocytes (arrowheads). B) Dog with DCM. All cardiomyocytes exhibit moderate cytoplasmic staining with an intensity similar to that seen in smooth muscle cells of small arterial walls (arrow). C) Dog with severe pulmonary stenosis. Bundles of weakly positive cardiomyocytes are seen adjacent to aggregates of negative cardiomyocytes (arrowheads). Arrow: small artery. D) Dog with brain tumour. Cardiomyocytes exhibit a weak cytoplasmic reaction. Arrow: small artery. Horseradish peroxidase method, Papanicolaou’s haematoxylin counterstain. Bar = 20 \( \mu \)m.

Comment [17]: The results were normalised for analysis (line 192-194). I added both, because that was suggested by the pathologists, who gave feedback after my presentation. I can remove one, but in that case possibly better the mean?
Fig. 3. Scatter plots showing a positive correlation between 5-HTR2B mRNA expression and IL-1 ($r = 0.719, P < 0.001$), MMP-13 ($r = 0.770, P < 0.001$) (3a), TGF-β ($r = 0.848, P < 0.001$) and TIMP-1 ($r = 0.729, P < 0.001$) (3b) mRNA concentrations.

Editor comment: is it worthwhile illustrating the correlation data, comparing 5-HTR2 with cytokine, MMP and TIMP values?
Figure 3a

Figure 3b