

Quantitative mRNA analysis of serotonin 5-HT₄ receptor isoforms, calcium handling proteins and ion channels in human atrial fibrillation

Frank Lezoualc'h^{a,b}, Klaudia Steplewski^c, Laura Sartiani^d, Alessandro Mugelli^d,
Rodolphe Fischmeister^{a,b,*}, Antoine Bril^{c,1}

^a INSERM, U769, F-92296 Châtenay-Malabry, France

^b Univ. Paris-Sud 11, IFR-141, F-92296 Châtenay-Malabry, France

^c GlaxoSmithKline Pharmaceuticals, King of Prussia, PA 19406-0939, USA

^d Dipartimento di Farmacologia Preclinica e Clinica, Università degli Studi, 50139 Firenze, Italy

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Abstract

Serotonin 5-HT₄ receptors are present in human atrial myocytes and have been proposed to contribute to the generation of atrial fibrillation (AF). Here, we quantified 5-HT₄ receptors as well as other key genes involved in cardiac rhythm and contraction in right atrial appendages of patients with chronic AF (CAF) and acute AF (AAF). Right atrial appendages were obtained from eleven patients in sinus rhythm (SR), five with AAF and six with CAF (>12 months). TaqMan real time quantitative RT-PCR was performed on total RNA. Results were normalised to the average of three housekeeping genes, cyclophilin, GAPDH and RL-19. The rank order of expression of h5-HT₄ receptors variants was (b) > (a) > (g) > (c) in the group of patients in SR. In AAF, we found a strong decrease in h5-HT_{4(b)}, h5-HT_{4(c)}, and h5-HT_{4(g)} transcripts. In CAF patients, the mRNA expression level of the h5-HT_{4(b)} isoform significantly increased two fold versus SR. A similar increase was reported for β_1 -adrenergic receptor, connexin 43 and the L-type Ca²⁺ channel CaC-NA1C subunit. Interestingly, CAF was associated with a strong increase in the expression of Na⁺/Ca²⁺ exchanger and the voltage-dependent Na⁺ channel SCN5A subunit. Our results indicate that h5-HT_{4(b)} is the dominant cardiac isoform of human 5-HT₄ receptors and its expression is increased in CAF. These data support the involvement of 5-HT₄ receptors in atrial arrhythmia.

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Atrial fibrillation (AF) is the most common sustained arrhythmia in the Western world and its prevalence is increasing with age [1]. AF is frequently observed in patients with hypertension and ischemic heart disease, being more common in patients having congestive heart

failure [2,3]. In addition, transient atrial fibrillation appears postsurgically in approximately one third of patients undergoing coronary artery bypass surgery [4]. Although some progress has been made in the therapy and prevention of atrial arrhythmia and particularly of atrial fibrillation, the use of antiarrhythmic drugs that modulate cardiac ion channels is associated with organ toxicity and potential ventricular proarrhythmic effects [5,6]. Consequently novel and safe therapeutic approaches need to be developed.

Electrophysiological remodelling is a central event in AF and is characterised by the shortening of the atrial effective refractory period (AERP). It is associated with

* Corresponding author. Address: INSERM, U769, Faculté de Pharmacie, Université Paris-Sud 11, 5 Rue Jean-Baptiste Clément, F-92296 Châtenay-Malabry, France. Fax: +33 1 46 83 54 75.

E-mail addresses: fisch@vjf.inserm.fr (R. Fischmeister), antoine.bril@fr.netgrs.com (A. Bril).

¹ Present address: IdRS, 11 rue des Moulineaux, F-92150 Suresnes, France. Fax: +33 1 55 72 26 40.

changes in gene expression and functional properties of key proteins involved in ion transport [2,7]. Therefore, one possible approach for the treatment of AF is the development of antiarrhythmic drugs which target membrane receptors specifically involved in the regulation of cellular electrophysiology of atrial cardiomyocytes. One such receptor which has gained attention for its involvement in AF is the serotonin 5-HT₄ receptor [8–10].

Several splice variants of the human 5-HT₄ receptor (h5-HT₄) have been described, particularly at the C-terminus where alternative splicing results in divergent sequences following Leu358 [10]. These h5-HT₄ receptor isoforms belong to the family of seven transmembrane domain G-protein coupled receptors (GPCRs) which activate adenylyl cyclase [10,11]. 5-HT₄ receptor activation leads to strong positive chronotropic, inotropic and lusitropic effects in human and pig atrium [8] through activation of the cAMP/PKA pathway and stimulation of several key components of the excitation–contraction coupling such as the L-type Ca²⁺ channel current ($I_{Ca,L}$) [12,13] and the hyperpolarization-activated pacemaker current I_f [14]. Although expressed at the mRNA level in human ventricle [15,16], 5-HT₄ receptor activation does not normally exert any effect on contractility [16,17] or $I_{Ca,L}$ [12] in this tissue. However, expression level of the 5-HT₄ receptor in the ventricle was recently found to increase in a rat model of heart failure [18] and this is accompanied by the appearance of a positive inotropic effect upon receptor activation [18,19]. This indicates that the 5-HT₄ receptor may undergo changes in its expression level and function depending on the pathophysiological state of the cardiac tissue.

The preferential location of the 5-HT₄ receptor in the atrium, together with its stimulatory effect on arrhythmogenic currents such as $I_{Ca,L}$ and I_f , makes it reasonable to assume that the 5-HT₄ receptor participates in the control of cardiac rhythmic and/or arrhythmic activity [8]. In support of this hypothesis, RS-100302, a 5-HT₄ receptor antagonist, inhibited AF induced by rapid pacing in juvenile pigs [20]. Thus, 5-HT₄ receptors may represent a new potential therapeutic target for the prevention or treatment of AF.

To date, there is no detailed information on the expression levels of the different h5-HT₄ receptor isoforms in human atrium, both in normal and pathological tissues. One major drawback of the study of 5-HT₄ receptor expression in human atrium is its very low density which renders its detection rather difficult by classical biochemical techniques [21]. In addition, specific antibodies against the different 5-HT₄ splice variants are not yet available. But most importantly, selective ligands for the different 5-HT₄ receptor isoforms are still lacking and this excludes the feasibility of any functional study which aims at elucidating the role of the different 5-HT₄ splice variants in native tissues [10]. In this study, we therefore performed the quantification of cardiac h5-HT₄ receptors transcripts by TaqMan reverse transcriptase-polymerase chain reaction

(RT-PCR). mRNA expression levels of the cardiac h5-HT₄ receptor C-terminal splice variants h5-HT_{4(a)}, h5-HT_{4(b)}, h5-HT_{4(c)}, and h5-HT_{4(g)} were quantified in right atrial appendages of patients with chronic AF (CAF) and acute AF (AAF) following coronary artery bypass grafting (CABG). Furthermore, the expression levels of a whole panel of key genes involved in cardiac rhythm and contraction were investigated.

Methods

The investigation conforms with the principles outlined in the Declaration Helsinki and was approved by the local ethical committee; informed consent was obtained before cardiac surgery. Patients underwent coronary artery bypass grafting (CABG, $n = 19$), CABG plus aortic valve replacement ($n = 2$) and mitral valve replacement ($n = 1$). Specimens of right atrial appendages were obtained as part of the routine atriotomy procedure and were immediately frozen in liquid nitrogen. Methods used for RNA preparation, real-time quantitative RT-PCR and statistical analysis are detailed in the online [Supplementary Data](#) to this article.

Results and discussion

Patients

Twenty two patients undergoing cardiac surgery were studied: all the patients were electrocardiographically monitored all over the period of hospitalization. They were divided in three different groups: sinus rhythm (SR), patients in SR who did not develop atrial fibrillation after cardiac surgery; acute atrial fibrillation (AAF), patients in SR who developed atrial fibrillation after surgery; chronic atrial fibrillation (CAF), patients with documented permanent atrial fibrillation from at least one year before cardiac surgery. Pre-surgical pharmacological therapy and the main clinical and demographic characteristics of the patients are given in [Table 1](#).

mRNA expression analysis of 5-HT₄ receptor isoforms in human atrial samples

We investigated the expression of h5-HT₄ receptor C-terminal splice variants in the three groups of patients either with sinus rhythm (SR, $n = 11$), or developing AAF ($n = 5$) or CAF ($n = 6$) ([Fig. 1](#)). The h5-HT_{4(b)} receptor isoform was consistently 15- to 23-fold higher than h5-HT_{4(c)} and h5-HT_{4(g)} receptors in SR group, which were quite similar to each other but a further 2- to 3-fold higher than h5-HT_{4(a)} ([Fig. 1](#)). As previously shown by non-quantitative RT-PCR [11], we were unable to detect any messenger encoding the h5-HT_{4(d)} receptor isoform in human atrial samples (data not shown). These results indicate that, like in the central nervous system and peripheral tissues such as the small intestine [22], the h5-HT_{4(b)} receptor isoform is the predominant splice variant expressed in human atria. We then compared the expression profile of h5-HT₄ receptor isoforms in the groups of AAF and CAF patients to the group of controls in SR ([Fig. 1](#)). We found a drop in

Table 1
Characteristics of the patients

	Sinus rhythm	Acute	Chronic	Acute + chronic
Total number	11	5	6	11
Sex (M/F)	11/0	5/0	4/2	9/2
Age (years)	67 ± 2	68 ± 4	70 ± 3	69 ± 2
Atrial fibrillation	Absent	Post-CABG	Permanent (>1 year)	
Cardiac disease				
Coronary artery disease	11	5	4	
Aortic valve disease	0	0	2	
Mitral valve disease	0	2 ^a	1	
Left ventricular function				
Normal	2	2	1	
Mild to moderate dysfunction	3	1	4	
Severe dysfunction	1	0	0	
Not available	5	2	0	
LVEF (%)	45 ± 5	55 ± 10	42 ± 5	45 ± 5
Hypertension (No. patients)	7	2	0	2
Medication (%)				
Diuretics	—	20	100	64
Digoxin	—	—	67	36
Antiarrhythmics	18	20	17	18
ACE-I/ARB	18	20	67	46
Nitro-vasodilators	82	100	33	64
Beta-blockers	27	60	17	36
Calcium antagonists	64	40	—	18
Anticoagulants	46	20	50	36
Aspirin	46	80	33	55

LVEF, left ventricular ejection fraction; ACE-I, angiotensin converting enzyme-inhibitors; ARB, angiotensin II receptor blockers.

^a Mitral insufficiency.

the expression levels of the h5-HT_{4(b)} splice variants in the group of AAF patients (Fig. 1). In this group, the rank order of expression of h5-HT₄ receptors variants was (a) = (b) = (c) > (g) indicating that the predominance of the (b) isoform in SR patients was completely lost in AAF patients. Regarding the group of CAF patients, we observed a significant increase in the expression level of the h5-HT_{4(b)} receptor isoform which was twofold higher than that observed in the SR group (Fig. 1). As observed in the group of patients in SR, the h5-HT_{4(b)} splice variant remained the predominant 5-HT₄ receptor isoform expressed in the CAF group, with a rank order of abundance for the different isoforms b > a = c > g (Fig. 1). The observed differences in 5-HT₄ receptor isoform levels were not due to differences in primer/probe characteristics since we have previously demonstrated that the primer/probe set for h5-HT_{4(b)} was slightly less efficient than the primer/probe sets for the other variants [22]. The observed up-regulation of the major 5-HT_{4(b)} receptor isoform in patients with CAF contrasts with work from Grammer and colleagues [23] who found a slight decrease (36%) in the amount of 5-HT₄ receptor in mRNA in atrial tissues of patients with CAF. A reason for this discrepancy could reside in the technique employed by the authors to quantify 5-HT₄ receptors. Indeed, they used semi-quantitative RT-PCR which is a much less sensitive technique than TaqMan RT-PCR performed in our study. Alternatively, the duration of AF could also be a factor that might influence the expression of 5-HT₄ receptors in the atrium. Indeed, Gram-

mer and colleagues [23] included patients who had CAF for more than one month whereas we selected patients affected by CAF for more than one year. In agreement with this hypothesis, we found a significant decrease in the expression of the 5-HT_{4(b)} receptors in patients with AAF following CABG.

mRNA expression of GPCRs and calcium handling proteins in AF

5-HT₄ receptors share some similarities with β -adrenergic receptors in their signalling cascade and their effects on ion channels [10,24]. Therefore, we analysed mRNA expression levels of β_1 -(β_1 -AR) and β_2 -(β_2 -AR) adrenergic receptors in right atrial appendages of patients in sinus rhythm, and with AAF or CAF (Fig. 2). In accordance with a previous study [23], we observed a significant increase in β_1 -AR expression in the group of CAF patients as compared to SR and AAF groups whereas no significant changes were detected for β_2 -AR transcripts between the three groups of patients (Fig. 2). Other GPCRs such as the M₂R and AT₁-R and AT₂-R receptors which have been shown to be involved in the structural remodelling occurring in AF [25], were also assayed by TaqMan real time RT-PCR (Fig. 2). Although we found a tendency for an increase in the expression of these genes in patients developing AAF *vs.* SR group, these changes did not reach statistical significance (Fig. 2). Furthermore, no changes in the mRNA expression of AT₁-R and AT₂-R were observed in

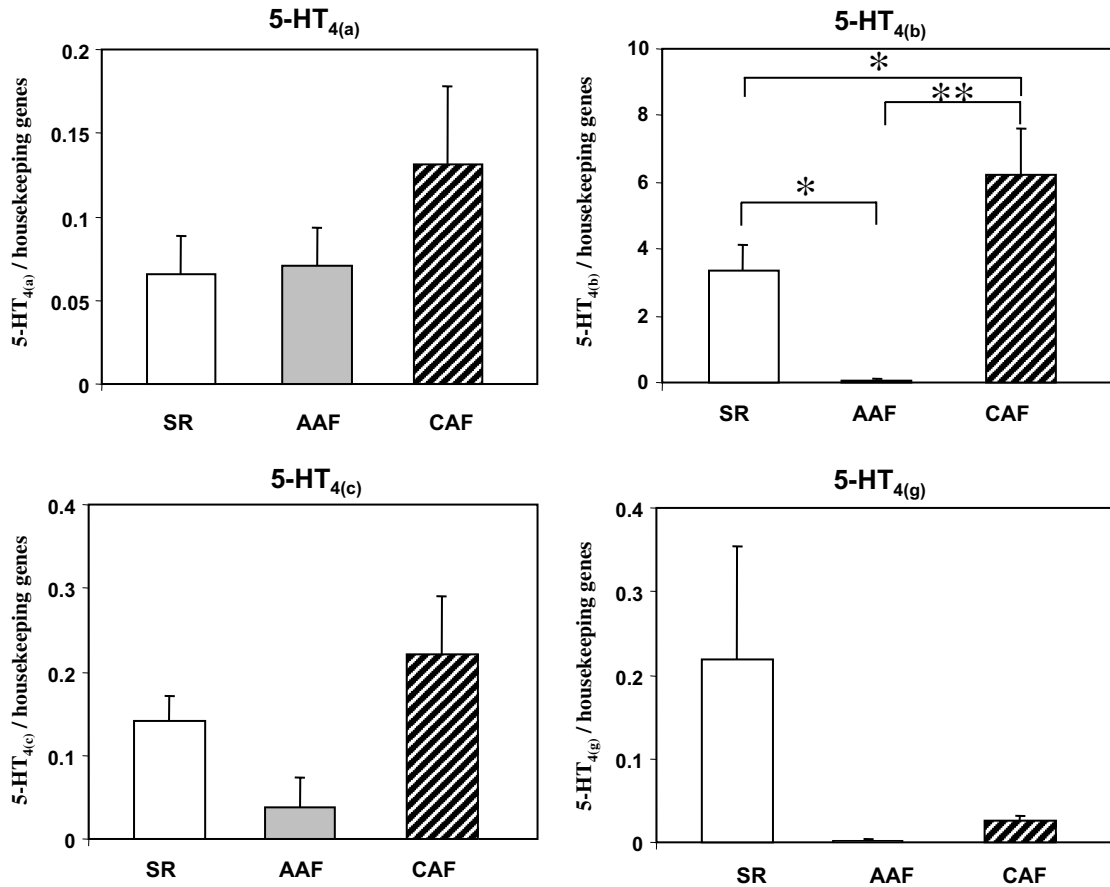


Fig. 1. 5-HT₄ receptor splice variant mRNA expression in right atrial appendages of patients with sinus rhythm, or developing acute or chronic atrial fibrillation. mRNA expression profiles are shown for the 5-HT_{4(a)}, 5-HT_{4(b)}, 5-HT_{4(c)}, and 5-HT_{4(g)} receptor isoforms. TaqMan RT-PCR analysis was performed using total RNA extracted from each patient. Data are expressed as arbitrary fluorescent units normalized to cyclophilin, GAPDH, and RL-19 to correct for RNA quantity and integrity, and are mean values \pm SEM. SR, sinus rhythm ($n = 11$); AAF, acute AF ($n = 5$); CAF, chronic AF ($n = 6$).

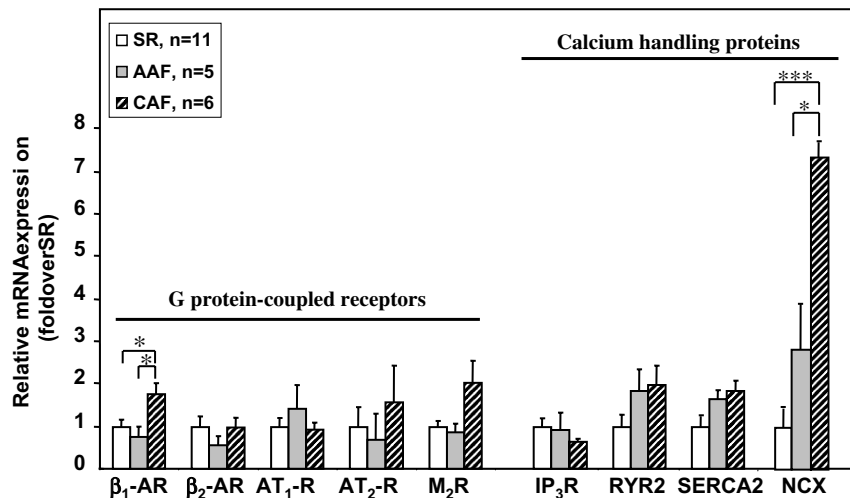


Fig. 2. Comparison of the expression levels of G-protein coupled receptors and calcium handling proteins in patients with sinus rhythm *versus* patients developing acute or chronic atrial fibrillation. mRNA expression of the different genes were determined by quantitative TaqMan RT-PCR using primer/probe sets as described in Supplementary Data Supplementary Table 1. Data are expressed as fold over sinus rhythm (SR) normalised to cyclophilin, GAPDH and RL-19. Results are mean values \pm SEM. AAF, acute AF; CAF, chronic AF.

CAF. This is supported by another study which has shown that the expression of AT-R proteins is regulated at post-transcriptional levels in AF [25].

Because cytosolic calcium abnormalities are important mediators of AF [26], gene expression of proteins influencing calcium handling was also investigated (Fig. 2).

Although it has been reported that AF may down regulate the expression of SERCA2a and up regulate the expression of 1,4,5-trisphosphate receptor (IP₃R), respectively [27–29], we did not find any significant changes in mRNA expression of SERCA2a, IP₃R or RYR2 between the group of SR, AAF, and CAF patients (Fig. 2). Interestingly, we detected a strong increase in the expression level of NCX in patients with CAF (Fig. 2). Indeed, expression level of NCX mRNA in the group of patients with CAF was more than sevenfold higher than that in SR (Fig. 2). The amount of NCX transcripts was also significantly increased in the group of AAF patients, albeit to a lower extent (Fig. 2). These results are in accordance with a recent study [30]. Under physiologic conditions, NCX extrudes intracellular Ca²⁺ during diastole [24]. In contrast, NCX is over expressed in heart failure and has been shown to contribute to both contractile dysfunction and arrhythmogenesis [31]. Therefore, one could speculate that the high increase in the expression of NCX observed in CAF in the present study might contribute to the induction and maintenance of atrial electrical remodelling and contractile dysfunction.

Gene expression of ion channels

CAF is associated with a sustained shortening of the atrial action potential duration and AERP [7,26]. AERP involves functional changes in ion channels, particularly

in repolarizing K⁺ channel activity [32,33]. We therefore investigated whether AAF and CAF are associated with any change in the expression levels of repolarizing outward K⁺ channel subunits such as KCN5 (Kv1.5), Kv4.3, and HERG. No significant changes were found for Kv1.5, Kv4.3, and HERG mRNA levels in either group (Fig. 3A). Interestingly, a significant reduction in a cardiac pacemaker channel, HCN4 was observed in the group of patients with CAF (Fig. 3A). Transcripts of two other members of this family, HCN1 and HCN2 were not significantly altered (Fig. 3A).

Besides repolarizing outward K⁺ channels, L-type Ca²⁺ channel reduction also contributes to shortening the AERP and action potential duration [32,34]. Unexpectedly, we found that CAF was associated with a significant increase in the mRNA amount of L-type Ca²⁺ channel α_{1C} subunit (CaCNA1C) as compared to the group in SR and AAF, whereas no changes were observed in the expression of T-type Ca²⁺ channel α_{1H} (CaCNA1H) and α_{1G} (CaCNA1G) subunits between the three groups of patients (Fig. 3B). The fact that we observed a simultaneous up-regulation of the major 5-HT_{4(b)} receptor isoform and the L-type Ca²⁺ channel α_{1C} subunit in patients with CAF might lead to excessive activation of I_{Ca,L} by 5-HT₄ receptors, causing intracellular calcium overload resulting in delayed after-depolarizations and triggered activity [8]. While our results contrast with previous findings showing that I_{Ca,L} amplitude as well as the gene

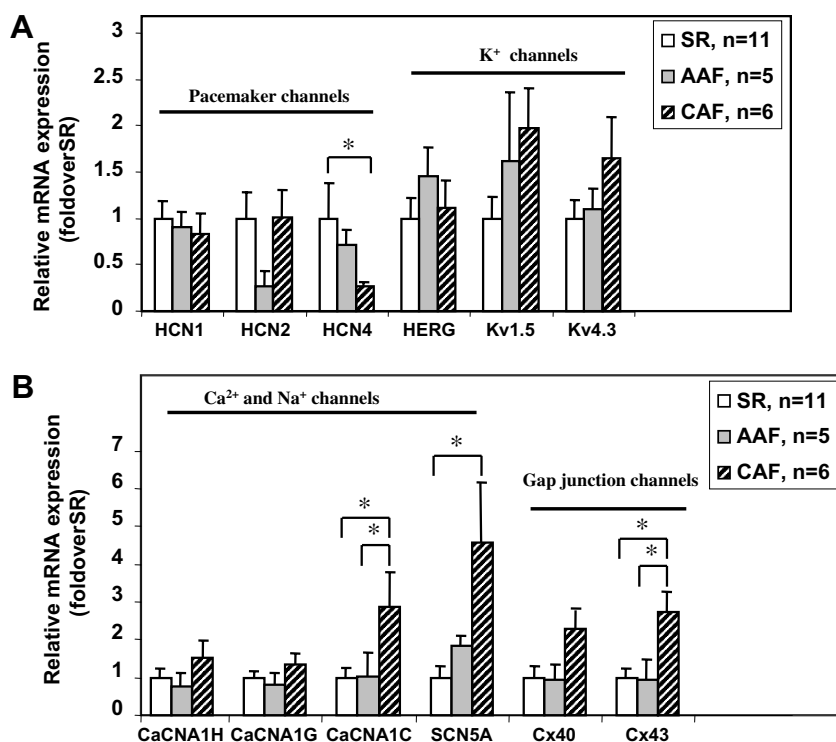


Fig. 3. mRNA expression analysis of ion channels in atrial fibrillation. Change in gene expression was analyzed by quantitative TaqMan RT-PCR. Histograms show the relative mRNA abundance of hyperpolarization-activated inward channels and outward K⁺ channels (A), and Ca²⁺ and Na⁺ inward and gap junction channels (B). Data are expressed as fold over sinus rhythm (SR) normalised to housekeeping genes. Results are mean values \pm SEM. AAF, acute AF; CAF, chronic AF.

expression of L-type Ca^{2+} channel α_{1C} subunit and HERG, and Kv4.3 are significantly reduced in CAF [27,28,35,36]. Schotten and colleagues [37] recently found no change in the protein expression of the L-type Ca^{2+} channel α_1 and β_2 subunits in CAF. Altogether, these observations suggest that the regulation of the L-type Ca^{2+} channel is particularly complex in AF.

Expression of the voltage-dependent Na^+ channel (I_{Na}) SCN5A subunit paralleled the observed increase in L-type Ca^{2+} channel (Fig. 3B), whereas it was found to be unaltered in another study on human AF [27]. Moreover, both the mRNA [34] and the density of I_{Na} [38,39] were found to decrease in a dog model of atrial fibrillation. Although the exact reasons for the discrepancies between the expression level of different ion channel genes in our study and those mentioned above are unknown, one could argue that these discrepancies may be due to methodological differences (TaqMan real time quantitative RT-PCR in our study *vs.* semi-quantitative RT-PCR in the other ones), differences in patients' medication and clinical parameters, in particular the duration of persistent AF (>1 year in this study *vs.* >1–3 months in others), and differences in AF models (human AF *vs.* dog model of AF induced by rapid-pacing).

Since altered expression of gap junction proteins (connexins) in diseased myocardial tissue may lead to abnormal electrical coupling between cardiomyocytes and hence contribute to arrhythmogenesis [40], we examined the expression of Cx40 and Cx43. Previous studies performed in animal models of AF reported contradictory results [40]. Although, Cx43 expression is increased in canine model of AF [41] it is unaltered in the goat [42]. Another study in human showed that Cx40 mRNA was expressed at significantly higher levels in patients who developed postoperative AF than those who did not [43]. In the same study, the amounts of Cx43 were not significantly different between the groups. Here, we found that Cx43 was significantly increased in the group of CAF as compared to SR patients (Fig. 3B) but no significant change in Cx43 expression level was observed in AAF patients.

In conclusion, we have shown that the 5-HT_{4(b)} receptor isoform is the most preponderant 5-HT₄ receptor splice variant expressed in human atrium. Furthermore, we found that serotonin 5-HT₄ receptor transcripts are differentially regulated in AAF and CAF. The upregulation of 5-HT_{4(b)} receptor isoform in CAF supports the involvement of 5-HT₄ receptors in atrial arrhythmia and the potential therapeutic utility of 5-HT₄ receptor antagonists in this cardiac disorder. The increase in I_{Na} channel expression together with an increased expression of NCX may reinforce the hypothesis that arrhythmogenesis in CAF is enhanced and could provide a molecular basis for the “AF begets AF” theory [2]. Further experiments are required to determine whether these proteins are modulated in a similar manner at the protein or functional level in AF. These await the production of specific antibodies against the different 5-HT₄ splice variants and selective ligands for the different 5-HT₄ receptor isoforms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bbrc.2007.03.124](https://doi.org/10.1016/j.bbrc.2007.03.124).

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