# Regulation of sinus node pacemaking and atrioventricular node conduction by HCN channels in health and disease

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**Contents**

**Abstract**

The funny current, $I_f$, was first recorded in the heart 40 or more years ago by Dario DiFrancesco and others. Since then, we have learnt that $I_f$ plays an important role in pacemaking in the sinus node, the innate pacemaker of the heart, and more recently evidence has accumulated to show that $I_f$ may play an important role in action potential conduction through the atrioventricular (AV) node. Evidence has also accumulated to show that regulation of the transcription and translation of the underlying *Hcn* genes plays an important role in the regulation of sinus node pacemaking and AV node conduction under normal physiological conditions - in athletes, during the circadian rhythm, in pregnancy, and during postnatal development - as well as pathological states - ageing, heart failure, pulmonary hypertension, diabetes and atrial fibrillation. There may be yet more pathological conditions involving changes in the expression of the *Hcn* genes. Here, we review the role of $I_f$ and the underlying HCN channels in physiological and pathological changes of the sinus and AV nodes and we begin to explore the signalling pathways (microRNAs, transcription factors, GIRK4, the autonomic nervous system and inflammation) involved in this regulation. This review is dedicated to Dario DiFrancesco on his retirement.

## 1. Introduction

The funny current, $I_f$, was first observed in the years from 1968 to 1982 by the groups at the University of Oxford and the National Institute for Physiological Sciences in Okazaki. It was first observed in the Purkinje fibres making up the ventricular conduction system (Noble & Tsien, 1968), where it was wrongly interpreted as a decaying outward current and referred to as $I_{K,2}$. Interestingly, Noble (Noble, 1960) had earlier postulated the presence of such a current in cardiac pacemaker tissue. It was later observed in the pacemaker of the heart, the sinus node (Brown & DiFrancesco, 1980; Yanagihara & Irisawa, 1980; Yanagihara *et al.*, 1980), where it was referred to as $I_f$ or $I_h$ (hyperpolarization-activated current). Finally, it was reported in the atrioventricular (AV) node, which is responsible for conduction of the action potential from the atria to the ventricles (Kokubun *et al.*, 1982). Therefore, $I_f$ is present throughout the cardiac conduction system (sinus node, AV node and His-Purkinje system). The hyperpolarization-activated cyclic nucleotide-gated (HCN) ion channels responsible for $I_f$ in the heart were identified ~20 years ago (Ludwig *et al.*, 1998; Ishii *et al.*, 1999; Ludwig *et al.*, 1999; Vaccari *et al.*, 1999) and since then they have been shown to be expressed throughout the cardiac conduction system. Since its discovery, $I_f$ has never been far from controversy. Early, there was the confusion about the ionic nature of $I_f$: first, it was considered to be a $K^+$ current, $I_{K,2}$ (Noble & Tsien, 1968), but later DiFrancesco correctly identified it as a mixed $Na^+/K^+$ current (DiFrancesco, 1981). After this, there was a debate concerning the importance of $I_f$ in pacemaking as compared to, first, background inward current (Noma *et al.*, 1983; DiFrancesco, 1991; Hagiwara *et al.*, 1992) and, secondly, the $Ca^{2+}$ clock (Lakatta & DiFrancesco, 2009). The debate concerning $I_f$ (part of the so-called membrane clock) and the $Ca^{2+}$ clock continues.

This review concerns yet another debate. In 1880, the University of Cambridge physiologist Walter Holbrook Gaskell wrote that most physiologists attributed the heartbeat to “the action of certain ganglion cells situated in the heart itself, while the cardiac muscular tissue is credited with the purely subordinate role of responding to the impulses generated in these nerve cells” (Gaskell, 1880). In other words, at this time, most physiologists believed the heartbeat to be neurogenic like the contraction of skeletal muscle, but Gaskell went on to disprove this and prove that the heart beat is myogenic in origin by showing that the apex of the tortoise heart although devoid of ganglion cells has the ability to beat rhythmically like other parts of the heart (Gaskell, 1883). ~20 years later in

1907, Keith and Flack discovered the natural pacemaker of the heart, the sinus node (Keith & Flack, 1907), and after a further ~60 years $I_f$ was discovered. It can be argued that the discovery of $I_f$ finally resolved the original neurogenic versus myogenic debate, which had been ongoing for ~1000 years – Galen, a physician, surgeon and philosopher in the Roman Empire, observed that the heart continued to beat after it had been excised and concluded that "the pulsative faculty of the heart has its source in its own substance" (Fye, 1987; Boyett & D'Souza, 2020). However, what about *changes* in heart rate? We know that short-term changes in heart rate such as *during* exercise, the vaso-vagal reflex or the diving reflex are 'neurogenic' and caused by the autonomic nervous system. However, there is a vigorous modern neurogenic versus myogenic debate concerning long-term changes in heart rate in the athlete and during the day-night cycle (Boyett & D'Souza, 2020). These changes are generally attributed to the vagus (i.e. they are neurogenic), but could they be myogenic, i.e. intrinsic to the heart? Long-term changes in heart rate occur in other circumstances as well, and what is responsible for them? This review shows an important role for myogenic mechanisms: an important role for HCN channels and $I_f$ in long-term heart rate changes both under physiological conditions (in athletes as well as during the circadian rhythm, pregnancy and postnatal development) and in pathophysiological states (in ageing, heart failure, pulmonary hypertension, diabetes and atrial fibrillation - although ageing is not in itself a pathological state, it is associated with sinus node dysfunction). This work raises questions about transcriptional regulation of HCN channels and this review also discusses this newly emerging field of study. Finally, this review highlights a new role for HCN channels and $I_f$ in atrioventricular (AV) node conduction. This review is dedicated to Dario DiFrancesco on the occasion of his retirement.

Although this review focusses on HCN channels, it does not mean that other ion channels and mechanisms are not involved, and some are mentioned here. In this review, proteins are written in capitals (e.g. HCN4) whereas mRNAs (transcripts) and genes are written in italics in lower case with an initial capital letter (e.g. *Hcn4*).

## 2. Expression of HCN channels in the sinus node and AV node

The sinus node is located at the junction of the superior vena cava with the right atrium. It is frequently shown in medical textbooks as a small nodule at this point, but is now known from the work of Dobrzynski and others to extend from the junction down the crista terminalis (a thick bundle

of atrial muscle) towards the inferior vena cava and is closely associated with the sinus node artery (Fig. 1A-D) (Dobrzynski *et al.*, 2005; Chandler *et al.*, 2011; Stephenson *et al.*, 2012; Stephenson *et al.*, 2017). There are four HCN channels. HCN1 channels have a more positive threshold for activation and faster activation kinetics. The cyclic nucleotide binding domain of HCN1 channels contains pre-bound cAMP (Lolicato *et al.*, 2011). This high affinity for cAMP of HCN1 channels renders HCN1-mediated $I_f$ poorly sensitive to variations in intracellular cAMP concentration (Lolicato *et al.*, 2011). In contrast, HCN4 channels are slowly gating and have higher sensitivity to variations in cAMP level. Finally, HCN2 and HCN3 have intermediate properties between HCN1 and HCN4 (Santoro & Tibbs, 1999; Baruscotti *et al.*, 2005; Wahl-Schott & Biel, 2009). Interestingly, cAMP induces channel tetramerisation of HCN2 and HCN4 channels (Lolicato *et al.*, 2011). HCN4 is widely regarded as the most highly expressed isoform in the two nodes. For example, in the mouse sinus node, the abundance of the channel transcripts as determined by RNAseq is *Hcn4>Hcn1>Hcn2>>Hcn3* (Wang *et al.*, 2021) and this is typical for the two nodes. The expression of HCN4 (green signal) and Cx43 (red signal) proteins in the rat sinus node as determined by Yanni and Dobrzynski using immunohistochemistry is shown in Fig. 1E (Boyett, 2009). Whereas HCN4 is expressed exclusively (or almost exclusively) in the sinus node, Cx43 is expressed exclusively (or almost exclusively) in the surrounding atrial node. Expression of HCN4 protein in the AV node has been shown by us (Dobrzynski *et al.*, 2003) as well as others.

**3. What HCN4 mutations tell us**

Naturally occurring mutations in HCN4 in the human show the importance of the channel for both the sinus and AV nodes. Loss-of-function mutations in human HCN4 cause asymptomatic and symptomatic sinus bradycardia and chronotropic incompetence (Schulze-Bahr *et al.*, 2003; Milanesi *et al.*, 2006; Hategan *et al.*, 2017) and also AV block (Zhou *et al.*, 2014b), whereas gain-of-function mutation causes inappropriate sinus tachycardia (Baruscotti *et al.*, 2015). Interestingly, loss of function mutations in HCN4 also result in atrial fibrillation (AF) (Duhme *et al.*, 2013; Macri *et al.*, 2014). It is known that AF causes sinus node dysfunction by downregulating HCN4 (see below) and, therefore, the interaction between HCN4 and AF appears to be two-way.

**4. Regulation of the HCN channels in health - resting bradycardia in athletes**

It is well known that athletes have a low resting heart rate – a resting bradycardia (Fig. 2A) (D'Souza *et al.*, 2015). Despite this, the athlete's heart can still provide sufficient cardiac output at rest – athletes have enlarged hearts with a greater stroke volume (Maron Barry & Pelliccia, 2006) and this will help offset the effect of the lower heart rate. Elite cyclists can have resting heart rates of ~30 beats/min (D'Souza *et al.*, 2015) (see also Fig. 2A). This bradycardia is particularly marked at night (Fig. 2B) (Northcote *et al.*, 1989). At night, athletes can have nocturnal pauses, which have been documented to be as long as 15 s in veteran athletes (Northcote *et al.*, 1989). Sinus node dysfunction is more common in veteran athletes and they are more likely to need a pacemaker implanted (Baldesberger *et al.*, 2008). As stated in the Introduction, the resting bradycardia in athletes for many decades has been attributed to high vagal tone (Aubert *et al.*, 2003). This assertion is primarily based on measurement of heart rate variability (Aubert *et al.*, 2003). Heart rate variability is widely used as an indirect measure of autonomic tone to the heart (Aubert *et al.*, 2003). Athletes have a greater heart rate variability, and this is said to be evidence of an increase in vagal tone (Aubert *et al.*, 2003). However, following Zaza and colleagues (Zaza & Lombardi, 2001), we have shown that heart rate variability is primarily influenced by the heart rate and we have argued that the increase in heart rate variability in athletes is the result of the resting bradycardia in these individuals (i.e. the resting bradycardia is not a 'consequence' of the increase in heart rate variability or at least what is thought to be the underlying factor, an increase in vagal tone) (Monfredi *et al.*, 2014). Another way to test the role of the autonomic nervous system in the resting bradycardia resulting from exercise training is to pharmacologically block cardiac autonomic tone. This has been carried out many times in both human athletes as well as animal models (Boyett *et al.*, 2013). The majority of the studies show that a resting bradycardia is still present after complete autonomic blockade – for example, in the horse (Betros *et al.*, 2013). In human athletes, the relative bradycardia is greater after autonomic blockade (Katona *et al.*, 1982; Boyett *et al.*, 2013; D'Souza *et al.*, 2017). Bahrainy *et al.* (Bahrainy *et al.*, 2016) concluded that both parasympathetic (vagal) and sympathetic tone is unchanged in human subjects after six months of exercise training. This evidence is inconsistent with the high vagal tone hypothesis to explain the resting bradycardia in athletes.

Using rodent models of exercise training, we showed that the resting bradycardia is still present after complete autonomic blockade and, in addition, the intrinsic beating rate of the isolated

(denervated) sinus node preparation taken from exercise trained animals is lower (D'Souza *et al.*, 2014). This points to an intrinsic mechanism being responsible, and quantitative PCR (qPCR) showed a downregulation of the transcripts for many ion channels, including the HCN channels, in the sinus node of exercise trained animals (D'Souza *et al.*, 2014). There is a downregulation of the principal HCN channel, HCN4, at both transcript and protein levels, and there is also a downregulation in the density of the corresponding funny current, $I_f$ (Fig. 2C) (D'Souza *et al.*, 2014). Finally, we showed that block of HCN channels and $I_f$ by $Cs^+$ or ivabradine abolishes the difference in the intrinsic beating rate of the isolated sinus node taken from exercise trained animals and control sedentary animals, and the difference in the resting heart rate of conscious or anaesthetised exercise trained animals and control sedentary animals (D'Souza *et al.*, 2014). This suggests that the resting bradycardia in exercise trained animals is the result of the downregulation of HCN channels and $I_f$. The same may be true in human athletes: we showed that the lower the resting heart rate, the smaller the effect of ivabradine and this is consistent with a downregulation of $I_f$ in human athletes (D'Souza *et al.*, 2017). However, other factors may also be involved – for example, D'Souza *et al.* (D'Souza *et al.*, 2014) observed a downregulation of other ion channel transcripts (apart from *Hcn4*) and Bidaud *et al.* (Bidaud *et al.*, 2020b) have reported a training-induced downregulation of L- and T-type $Ca^{2+}$ currents, $I_{Ca,L}$ and $I_{Ca,T}$ (as well as $I_f$).

What is responsible for the downregulation of HCN4 and $I_f$ following exercise training? In recent years, we have focused on epigenetic regulation by microRNAs, small non-coding RNAs that regulate gene expression by either targeting a mRNA for degradation or by inhibiting translation (D'Souza *et al.*, 2017). A screen of microRNAs in the mouse sinus node identified significant increases in 25 microRNAs, for example miR-423-5p (Fig. 2D), following exercise training and some of these were suggested by bioinformatics and *in vitro* luciferase reporter gene analysis to target HCN4 (D'Souza *et al.*, 2017). miR-423-5p was identified as the most likely microRNA to be targeting HCN4. An antimiR targeting miR-423-5p given to mice *in vivo* prevented the exercise training-induced upregulation of miR-423-5p, downregulation of HCN4, and decrease in heart rate (Fig. 2D) (D'Souza *et al.*, 2017). Further experiments suggested that miR-423-5p was under the control of the transcription factor, NKX2.5 (D'Souza *et al.*, 2017).

Although miR-423-5p-NKX2.5 interactions may be the immediate cause of the exercise training-induced downregulation of HCN4, D'Souza *et al*. (D'Souza *et al.*, 2017) did not identify the pathway upstream of the microRNA. A clue about the possible upstream pathway came from an unexpected direction. In the sinus node, ACh released from the vagus binds to the muscarinic $M_2$ receptor and activates an outward $K^+$ current, $I_{K,ACh}$, carried by heteromultimeric channels comprised of Kir3.1 (GIRK1) and Kir3.4 (GIRK4); activation of $I_{K,ACh}$ results in an acute bradycardia, i.e. immediate slowing of heart rate (left hand side of Fig. 3A). Surprisingly, Mangoni and colleagues discovered that genetic ablation (i.e. knocking out) of Kir3.4 or pharmacologic block of $I_{K,ACh}$ by administration of the peptide blocker tertiapin-Q can rescue sinus node dysfunction and high-degree AV block caused by loss-of-function of inward current carrying ion channels, HCN4 and $Ca_v1.3$ (Mesirca *et al.*, 2014; Mesirca *et al.*, 2016; Bidaud *et al.*, 2020a). These findings were explained by the hypothesis that a decrease in inward current in diastole can be compensated for by decreasing an outward current, $I_{K,ACh}$, in diastole. Mesirca, Mangoni and colleagues asked the question whether knockout of Kir3.4 can reverse exercise training-induced bradycardia (Bidaud *et al.*, 2020b). Knockout of Kir3.4 did indeed reverse exercise training-induced bradycardia (Bidaud *et al.*, 2020b). However, D'Souza and colleagues showed that knockout of Kir3.4 prevented: (i) the upregulation of miR-423-5p (Fig. 3B) and other microRNAs likely to be targeting HCN4, (ii) the downregulation of HCN4 (Fig. 3C-E) and (iii) the downregulation of $I_f$ density with exercise training (Bidaud *et al.*, 2020b). We tentatively suggest that the ACh-activated $K^+$ channel may act as a signalling molecule to bring about the downregulation in HCN4 (Fig. 3A). However, there must be a stimulus acting on the ACh-activated $K^+$ channel following exercise training to bring about the transcriptional remodelling. One possibility is high vagal tone in athletes (Fig. 3A), although Bidaud *et al*. (Bidaud *et al.*, 2020b) obtained no evidence of this.

Knockout of Kir3.4 not only prevented the training-induced downregulation of $I_f$, it also prevented a training-induced downregulation of L- and T-type $Ca^{2+}$ currents in the sinus node as well as the training-induced prolongation of the PR interval and physiological hypertrophy (Fig. 3A) (Bidaud *et al.*, 2020b). Clearly, more work is required to unravel the mechanisms and pathways involved.

**5. Regulation of the HCN channels in health - circadian rhythm in heart rate**

It has been known since the 1600's that there is a circadian rhythm in the heart rate (Lemmer, 2009) – the heart rate is higher during the awake period and lower during the sleep period. Fig. 2B shows an example from Northcote *et al.* (Northcote *et al.*, 1989) in human athletes and sedentary controls. The heart rate is presumably higher during the awake period to anticipate the increase in demand for cardiac output during the awake period. Although it is known that increased heart rate is a predictor of all-cause and cardiovascular mortality, in the study of Johansen *et al.* (Johansen *et al.*, 2013) of middle-aged subjects with no apparent heart disease, only night-time (not day-time or 24 h) heart rate was associated with adverse cardiovascular events after adjusting for cardiovascular risk factors. We have worked on the circadian rhythm in heart rate for about seven years and it has proved to be the most difficult project we have worked on. Partly, this was because the circadian rhythm in heart rate is complex and multifactorial and involves many systems (not just the heart) - it is an excellent example of integrative whole animal physiology. However, partly, it was because the circadian rhythm in heart rate had already been attributed to a circadian rhythm in cardiac vagal tone for many decades (Boas & Weiss, 1929; Sutherland, 1929; Bexton *et al.*, 1986; Black *et al.*, 2019) and, therefore, the work proved controversial and it provokes strong opinions from reviewers and others.

The commonly held assumption is that vagal tone is high during sleep and this results in greater release of ACh, which via the muscarinic $M_2$ receptor, results in a greater activation of $I_{K,ACh}$ and possibly a lower basal intracellular cAMP concentration leading to a slower heart rate through the workings of the coupled clock system (Monfredi *et al.*, 2013). Heart rate variability has been used as one line of evidence to assert that the circadian rhythm in heart rate is the result of high vagal tone during sleep. Heart rate variability is high during sleep when heart rate is low (Vandewalle *et al.*, 2007). Once again, we argue that the heart rate variability may be high because the heart rate is low (and not vice versa). Another test used is complete autonomic blockade using pharmacological blockers. The data obtained are variable with some studies showing that the circadian rhythm in heart rate is unaffected by complete autonomic blockade and others showing that it is affected (Black *et al.*, 2019). We have previously reviewed the effect of complete autonomic blockade on the circadian rhythm in heart rate and tentatively conclude that acute autonomic blockade does not block the circadian rhythm in heart rate (Black *et al.*, 2019). The circadian rhythm in heart rate also persists

after chronic functional autonomic blockade: (i) knockout of the muscarinic $M_2$ ACh receptor or knockout of the $\beta_1$, $\beta_2$ and $\beta_3$ receptors mediating the effects of ACh and catecholamines released by the autonomic nervous system on the heart does not block the circadian rhythm in heart rate (although this does not rule out a role for neuropeptides released from vagal nerve endings) (Swoap *et al.*, 2008); and (ii) heart transplant patients with denervated hearts show a circadian rhythm in heart rate (although this does not rule out a role for a circadian variation in the level of circulating catecholamines) (Idema *et al.*, 1994; Kotsis *et al.*, 2005). However, the effects of chronic intervention should be viewed cautiously, because chronic intervention may lead to compensatory transcriptional remodelling. One problem has been that, in both the case of athletic bradycardia and the circadian rhythm in heart rate, imperfect surrogates of cardiac autonomic tone have been measured rather than autonomic tone itself. However, Bussey (Bussey, 2019) has recently directly recorded cardiac sympathetic and parasympathetic (vagal) nerve activity in the rat and observed no evidence of a circadian variation.

Our work has focussed on the mouse. We confirmed that there is a circadian rhythm in heart rate *in vivo*, and *in vivo* the circadian rhythm in heart rate persists after acute pharmacological autonomic blockade and on right cervical vagotomy (in rats) (D'Souza *et al.*, 2021). There is also a circadian rhythm in the intrinsic beating rate of the isolated and therefore denervated sinus node (D'Souza *et al.*, 2021). This work together with the work of others reviewed above suggests that there is an intrinsic circadian rhythm in sinus node pacemaking. There are circadian rhythms of many physiological systems and, ultimately, the majority, if not all of these, are the result of a molecular circadian clock (Patke *et al.*, 2020). There is a master circadian clock in the suprachiasmatic nucleus in the hypothalamus and there are peripheral clocks in many if not all peripheral tissues (Mohawk *et al.*, 2012). There is known to be a local circadian clock in the heart (Durgan & Young, 2010). Measurement of selected transcripts using qPCR by D'Souza *et al*. (D'Souza *et al.*, 2021) showed that there is a functioning local circadian clock in the sinus node (Fig. 4A); this was confirmed by coupling promoter activity of the clock gene *Period* to a bioluminescent reporter. qPCR, western blot and patch clamp showed that there is a circadian rhythm in HCN4 transcript and protein and the density of $I_f$ (Fig. 4B-D) (D'Souza *et al.*, 2021). Is the circadian rhythm in HCN4 and $I_f$ density responsible for the circadian rhythm in heart rate? Block of HCN channels and $I_f$ *in vivo* using

ivabradine (Fig. 4E) or *in vitro* (in the isolated sinus node) using 2 mM $Cs^+$ abolished the circadian rhythm in heart rate and intrinsic beating rate, respectively, and this suggests that the circadian rhythm in HCN4 and $I_f$ density is involved (D'Souza *et al.*, 2021).

In a subsequent study, we showed that ~44% of the sinus node transcriptome (7,134 of 16,387 transcripts) measured using RNA-seq exhibits a circadian rhythm (Wang *et al.*, 2021). Consistent with the previous study, *Hcn4* tended to show a circadian rhythm, but in addition, *Hcn1* and *Hcn2* showed significant circadian rhythms (Wang *et al.*, 2021). Regulators of HCN4 - TRPM7, a divalent-permeant channel-kinase, AMP kinase, and phosphoinositide 3-kinase (Sah *et al.*, 2013; Yavari *et al.*, 2017; Lin *et al.*, 2019) - also showed a circadian rhythm (Wang *et al.*, 2021). However, other pacemaker transcripts (for example $Ca^{2+}$ clock transcripts) showed a circadian rhythm (Wang *et al.*, 2021). Therefore, $I_f$ may not be the only pacemaker mechanism involved in the circadian rhythm in pacemaking. Transcripts responsible for receptors and signalling pathways known to control pacemaking, transcripts from genes identified by GWAS as determinants of resting heart rate, and transcripts from genes responsible for familial and acquired sick sinus syndrome all exhibited a circadian rhythm (Wang *et al.*, 2021).

In conclusion, based on the information presented above, there is little or no evidence that the circadian rhythm in heart rate is the result of the autonomic nervous system and *acute* regulation of ionic conductances as previously thought. Instead, there is evidence that the circadian rhythm in heart rate is the result of transcriptional regulation of ion channel expression. What is responsible for the circadian rhythm in transcripts? There are three possibilities:

*(i) The local circadian clock in the sinus node.* BMAL1 and CLOCK are important circadian clock transcription factors that exert transcriptional control over clock-controlled genes by forming a heterodimer and binding to enhancer (E-box) elements (Monfredi & Lakatta, 2019). On the basis of computational predictions, we identified eight E-box binding regions on the *Hcn4* gene; a ChIP assay confirmed that BMAL1 can bind to two specific intronic regions of the *Hcn4* gene (D'Souza *et al.*, 2021). It is therefore possible that the local circadian clock in the sinus node is involved in *Hcn4* transcription. However, current understanding of clock-controlled gene regulation is based on a co-operative action between core clock transcription factors and local, tissue-specific transcription factors (Beytebiere *et al.*, 2019). Therefore, it is likely that candidates among the 347 transcription

factors that we have identified to show a circadian rhythm in the sinus node (Wang *et al.*, 2021) are involved. microRNAs can also regulate expression as discussed above and Anderson *et al*. (Anderson *et al.*, 2019) have shown a circadian rhythm in 74 microRNAs. We are exploring specific targeting of *Hcn4* by rhythmic microRNAs.

After cardiomyocyte-specific knock out of the key circadian clock gene, *Bmal1*, in the mouse, Schroder *et al*. (Schroder *et al.*, 2013) have shown that the circadian rhythm in heart rate continues almost unchanged. At face value, this is evidence that the local circadian clock in the heart does not play a major role in the circadian rhythm in heart rate; a contemporary review on the subject has also arrived at this conclusion (Rana *et al.*, 2020). However, Anderson, Boyett and D'Souza using RNAseq (Wang *et al.*, 2021) have shown that in the sinus node of a cardiomyocyte-specific *Bmal1* knockout mouse (D'Souza *et al.*, 2021), *Bmal1* is not knocked out but only marginally depressed and the other clock genes continue to oscillate almost unchanged (transcripts measured in n=3 knockout mice and n=3 wild-type mice at six time points over 24 h). Therefore, the continuing circadian rhythm in heart rate in the cardiac-specific *Bmal1* knockout mouse does not necessarily rule out the involvement of the local circadian clock in the sinus node. This uncertainty needs to be resolved. The circadian rhythm in heart rate also continues almost unchanged in a cardiac-specific *Clock* dominant negative mutant mouse (Bray *et al.*, 2008). Is this evidence against the involvement of the local circadian clock in the sinus node in the circadian rhythm in heart rate? Bearing in mind the experience of the *Bmal1* knockout mouse, it needs to be confirmed that the dominant negative *Clock* mutant does indeed abolish the local circadian clock in the sinus node myocytes. It may also be relevant that both *Bmal1* and *Clock* have paralogs (*Bmal2* and *Npas2*) that can substitute for *Bmal1* and *Clock* (Shi *et al.*, 2010; Landgraf *et al.*, 2016).

*(ii) The master clock in the suprachiasmatic nucleus.* Tong *et al*. (Tong *et al.*, 2013) have shown a circadian rhythm in various $K^+$ channels in mouse atria and ventricles and the circadian rhythm was abolished by *chronic* pharmacological blockade of the autonomic nervous system; they reported similar findings for connexins (Tong *et al.*, 2016). Tong *et al*. (Tong *et al.*, 2013) concluded that the master circadian clock in the suprachiasmatic nucleus is responsible for the circadian rhythm in the expression of the ion channels in the atria and ventricles. Tong *et al*. (Tong *et al.*, 2013) also reported that *chronic* pharmacological blockade of the autonomic nervous system abolished the

circadian rhythm in heart rate. Therefore, it is possible that the circadian rhythm in heart rate involves the autonomic nervous system and transcriptional regulation of ion channels.

In favour of master clock control are the findings that lesioning the suprachiasmatic nucleus completely abolishes the circadian rhythm in heart rate (as well as body temperature and physical activity rhythms), chronic autonomic blockade can abolish the circadian rhythm in rhythmic cardiac ion channel gene transcription, and the presence of an anatomical connection between the suprachiasmatic nucleus and the stellate ganglion - the main sympathetic ganglion innervating the heart (Ueyama *et al.*, 1999; Scheer *et al.*, 2003; Tong *et al.*, 2013; Tong *et al.*, 2016). Interestingly, depletion of peripheral catecholamine content with guanethidine abolished the circadian rhythm in heart rate specifically (body temperature and physical activity were unaffected) in the work of Warren et al. (Warren *et al.*, 1994), although this conclusion is disputed (Makino *et al.*, 1997; Oosting *et al.*, 1997). These data suggest that the master clock may signal to the sinus node via the sympathetic nervous system to bring about changes in gene transcription (Warren *et al.*, 1994).

*(iii) The master clock in the suprachiasmatic nucleus acting via the local circadian clock in the sinus node.* There are two ways by which the master clock in the suprachiasmatic nucleus may be generating circadian rhythms in transcripts in the sinus node. The autonomic nervous system may be directly controlling cardiac gene transcription, but alternatively the function of the autonomic nervous system may be to entrain the local circadian clock in the sinus node to the master circadian clock in the suprachiasmatic nucleus and it is the local clock that controls gene transcription. For example, it is known that β-agonist increases PER2 circadian rhythms in cultured cardiac ventricular tissue (Beesley *et al.*, 2016).

**6. Regulation of the HCN channels in health - increase in heart rate in pregnancy**

A variety of changes in the cardiovascular system occur during normal pregnancy in the human, including an increase in heart rate (Fig. 5A), stroke volume and cardiac output (Hall *et al.*, 2011). The increase in cardiac output is necessary to meet the increased metabolic demands of tissues and this is achieved by the increases in heart rate and stroke volume (Hall *et al.*, 2011). During pregnancy, there is an increased risk of arrhythmia, and a higher resting heart rate is a known risk factor for arrhythmias (El Khoury *et al.*, 2013). El Khoury *et al.* (El Khoury *et al.*, 2013) observed a faster heart rate in the pregnant mouse and this persisted in the absence of autonomic nervous

innervation: in the Langendorff-perfused heart and the isolated sinus node cell. Pregnancy increased the density of $I_f$ (Fig. 5C) and, although there was no change in the expression of HCN4, there was an increase in the expression of HCN2 (El Khoury *et al.*, 2013). El Khoury *et al.* (El Khoury *et al.*, 2013) concluded that the increase in $I_f$ density contributes to the increase in heart rate in pregnancy. The increase in $I_f$ during pregnancy is caused by 17β-estradiol (Long & Fiset, 2020).

**7. Regulation of the HCN channels in health - postnatal development**

The heart rate of the human and other large mammals decreases postnatally. In the human, it decreases from ~140 to 70 beats/min (Fig. 5B). In the rabbit, we showed that there is a decrease in the intrinsic heart rate (measured in the isolated sinus node) from the neonate to the adult (Abd Allah *et al.*, 2011). During postnatal development, qPCR revealed a significant decline in the sinus node of *Hcn1* and *Hcn4* mRNAs as well as other ion channel transcripts expected to impact pacemaking (Nav1.5, Cav1.3, *Slc8a1*/NCX1, Kv1.5, KvLQT1, minK and ERG; immunohistochemistry also showed complex changes in the expression of $Ca^{2+}$-handling proteins) (Abd Allah *et al.*, 2011). In the adult rabbit as compared to the newborn rabbit, Accili *et al*. (Accili *et al.*, 1997) observed a decrease in the maximal conductance of $I_f$ consistent with the findings of Abd Allah *et al.* (Abd Allah *et al.*, 2011). Accili *et al*. (Accili *et al.*, 1997) also observed a steeper dependence of activation of $I_f$ on membrane potential. The changes in *Hcn1, Hcn4* and $I_f$ could contribute to the decreased heart rate in the adult and this was confirmed by Zhang and colleagues using biophysically-detailed computer modelling (Alghamdi *et al.*, 2020b). However, this is unlikely to be the only factor involved: Baruscotti *et al*. (Baruscotti *et al.*, 1996) showed that in the rabbit the density and frequency of occurrence of the $Na^+$ current, $I_{Na}$, decreased during postnatal development and, whereas block of $I_{Na}$ by TTX decreased the spontaneous rate of sinus node cells from newborn rabbits by 63%, it had no effect on cells from adult animals. There is also a postnatal decease in the density of $I_{Na}$ in the dog (Protas *et al.*). This is perhaps consistent with the postnatal decrease in Nav1.5 mRNA described above, although sinus node cells also express neuronal type $Na^+$ channels (Protas *et al.*; Baruscotti *et al.*, 1997). There is also a postnatal decrease in the density of $I_{Ca,L}$ in the rabbit, although changes in the voltage-dependence of the current predisposes towards an increase rather than decrease in the contribution of $I_{Ca,L}$ to pacemaking (Protas *et al.*, 2001).

Interestingly, the heart rate of small mammals increases rather than decreases postnatally.

Adachi *et al*. (Adachi *et al.*, 2013) reported that in the mouse the heart rate increases from ~320 beats/min at birth to ~690 beats/min at postnatal day 14. In this case, the change was not associated with any change in $I_f$. Adachi *et al*. (Adachi *et al.*, 2013) concluded that the postnatal increase in heart rate is in part the result of an increase in sympathetic tone and in part an increase in the intrinsic heart rate (measured *in vivo* after autonomic blockade or *in vitro* in the isolated sinus node). In patch clamp experiments, there was an increase in the density of $I_{Ca,L}$ and a hyperpolarizing shift of the $I_{Ca,L}$ activation curve and this could be responsible for the postnatal increase in the intrinsic heart rate (Adachi *et al.*, 2013).

**8. Regulation of the HCN channels in disease - ageing**

Sinus node dysfunction is primarily a disease of ageing and it increases in an exponential-like manner with age (Fig. 6C) (Benditt *et al.*, 1995); AV node dysfunction increases in a similar fashion (Penton *et al.*, 1956). For example, pacemakers are mainly implanted in the elderly (https://www.statista.com/statistics/982630/number-of-new-pacemaker-implants-in-sweden-by-gender-and-age/). Although the resting heart rate in the human changes little with age after adulthood is achieved (Fig. 6A) (Kuga *et al.*, 1993; Ostchega *et al.*, 2011; Avram *et al.*, 2019), the heart rate *in vivo* is determined by the intrinsic properties of the sinus node together with the autonomic nervous system. Jose and Collison (Jose & Collison, 1970) measured the intrinsic heart rate after complete autonomic blockade in 432 human subjects and unlike the normal heart rate there was a steady decline of the intrinsic heart rate with age; Kuga *et al*. (Kuga *et al.*, 1993) obtained similar results (Fig. 6B). In addition, there is an age-dependent increase in the sinus node conduction time (time taken for the action potential, once initiated, to exit the sinus node) (Kuga *et al.*, 1993). This suggests that there is a decline in intrinsic sinus node function with age. This age-dependent decline has been the focus of various studies from us and others and the results have been varying. The oldest explanation is that age-dependent sinus node dysfunction is the result of fibrosis (i.e. a proliferation of the extracellular matrix). For example, interstitial collagen deposition and an increase in fibroblast area has been reported in the ageing mouse (Hao *et al.*, 2011; Moghtadaei *et al.*, 2016). The case for age-dependent sinus node fibrosis has been recently reviewed by Csepe *et al*. (Csepe *et al.*, 2015). However, Alings *et al*. (Alings *et al.*, 1995) investigated the possibility of age-dependent fibrosis in 41 human and 21 cat hearts and concluded that there is no age-dependent change in

collagen content (although there is a change in collagen organisation). In the ageing rat, Yanni *et al*. (Yanni *et al.*, 2010) observed no evidence of age-dependent fibrosis. For example, using qPCR, they observed a ~79% downregulation of mRNA for collagens 1 and 3 and a ~52% downregulation of mRNA for elastin (Yanni *et al.*, 2010). In contrast, an age-dependent increase in the interstitium and a loss of nodal cells has been reported in the rat (de Melo *et al.*, 2002).

The action potential in the centre of the sinus node has a slow upstroke because it is generated by the relatively small and slow L-type $Ca^{2+}$ current, $I_{Ca,L}$, rather than the much larger and faster $Na^+$ current as in the atrial and ventricular working myocardium. Alings and Bouman (Alings & Bouman, 1993) investigated the ageing rabbit and cat sinus node and showed that there is an enlargement of the area with a slow action potential. This is consistent with the loss of $Na^+$ channels and $I_{Na}$ from the sinus node - although $Na^+$ channels and $I_{Na}$ are absent from the centre of the sinus node, they are present in the periphery (Tellez *et al.*, 2006). Consistent with this, an age-dependent loss of mRNA for the cardiac $Na^+$ channel, Nav1.5, has been observed in the mouse sinus node (Hao *et al.*, 2011); an age-dependent loss of mRNA for other $Na^+$ channel isoforms has also been reported from the rat sinus node (Huang *et al.*, 2015). Zhang *et al*. (Zhang *et al.*, 1998) have shown using biophysically-detailed computer modelling that a decrease in $I_{Na}$ in the periphery of the sinus node would indeed lead to a slowing of pacemaking and an increase in the sinus node conduction time. This is supported by the fact that loss-of-function mutations of Nav1.5 are associated with sinus node dysfunction (Butters *et al.*, 2010). In the ageing rat, although we did not observe a decrease in Nav1.5 mRNA in the sinus node measured using qPCR (Tellez *et al.*, 2011), using immunohistochemistry, we did observe an expansion of the Nav1.5-negative area of the sinus node (Yanni *et al.*, 2010).

In studies of the ageing guinea-pig, using western blot, we observed a downregulation of protein for the gap junction channel, Cx43, and the L-type $Ca^{2+}$ channel, Cav1.2, in the sinus node; there was also an age-dependent increase in the sensitivity to $Ca^{2+}$ channel block by nifedipine consistent with a downregulation of $Ca^{2+}$ channels (Jones *et al.*, 2004; Jones *et al.*, 2007). However, in the ageing rat (Tellez *et al.*, 2011), we observed no changes in Cx43 and Cav1.2 mRNA measured by qPCR, but we did observe a significant downregulation of mRNA for the ryanodine receptor,

RyR2, which is known to play a central role in the $Ca^{2+}$ clock mechanism of pacemaking (Lakatta *et al.*, 2010).

In the ageing rat, we observed a large significant downregulation of *Hcn1* mRNA, but not of other *Hcn* mRNAs (Tellez *et al.*, 2011), and, in the ageing mouse, we observed a significant downregulation of *Hcn1* and *Hcn2* mRNAs (Hao *et al.*, 2011). In the ageing rat, Huang *et al*. (Huang *et al.*, 2007) observed a downregulation of *Hcn2* and *Hcn4* mRNAs. One of the most interesting studies of the ageing sinus node was carried out on the mouse by Larson *et al.* (Larson *et al.*, 2013). Using isolated sinus node myocytes, Larson *et al*. (Larson *et al.*, 2013) showed an age-dependent decrease in the density of $I_{Ca,L}$, $I_{Ca,T}$ and $I_f$ as well as a hyperpolarizing shift of the voltage-dependence of $I_f$; the decrease in $I_f$ density is shown in Fig. 6D,E. Interestingly, Sharpe *et al*. (Sharpe *et al.*, 2017) showed that, in isolated sinus node cells from mice, the effects of ageing were abolished by a high concentration of exogenous cAMP, which restored the beating rate and $I_f$ of sinus node cells from old mice to the level seen in cells from young mice.

In conclusion, age-dependent sinus node dysfunction is likely to be the result of sinus node remodelling including downregulation of HCN channels and $I_f$. However, disparate results have been obtained in different studies and the reason for this is unclear. One explanation for the variability amongst studies may be the presence of comorbidities and as a result the remodelling can take different forms. For example, our study of the ageing rat was carried out on males, and the male rats became obese with age (Yanni *et al.*, 2010). Moghtadaei *et al.* (Moghtadaei *et al.*, 2016) studied the effect of ageing in the mouse and noted that sinus node dysfunction is correlated with frailty (accumulation of health deficits), although the $R^2$ value was generally low and therefore frailty is a relatively poor indicator (for example, for *in vivo* heart rate versus frailty, $R^2$=0.09). Using a biophysically-detailed model of the rat sinus node action potential, Alghamdi *et al*. (Alghamdi *et al.*, 2020a) examined two different patterns of ion channel remodelling that have been reported in the aged rat sinus node and concluded that both patterns result in bradycardia; this work suggested that in each case it is the change in $I_{Ca,L}$ that plays the most important role and $I_f$ plays little or no role. However, there is experimental evidence that the downregulation of $I_f$ does play a role: Huang *et al*. (Huang *et al.*, 2007) reported that the intrinsic heart rate of the rat (measured *in vivo* after autonomic blockade decreases by 55 beats/min (from ~310 to ~255 beats/min) from the adult to the senescent

animal, and block of $I_f$ by 3 μM ivabradine prolonged the cycle length of the isolated sinus node from adult and senescent animals by ~26 and ~13%. This suggests that after partial block of $I_f$ (3 μM ivabradine causes ~50% block) the heart rate decreases by ~64 beats/min in the adult animal and only ~29 beats/min in the senescent animal, i.e. the age-dependent downregulation of $I_f$ does contribute to the age-dependent decrease in heart rate.

Clearly, further studies are required to resolve this confusing picture and D'Souza, Boyett and Smith have recently used RNAseq (Wang *et al.*, 2021) to study the transcriptome of the sinus node in ~2 and ~24 month-old male C57BL/6J mice (n=8/8). 55,401 transcripts were detected and there were significant changes in 7,206 of them. Fig. 6F shows a significant downregulation of transcripts underlying $I_f$ (*Hcn1*, *Hcn2*, *Hcn4*), $I_{Ca,L}$ (*Cacna1d*), $I_{Ca,T}$ (*Cacna1g*, *Cacna1h*) and the $Ca^{2+}$ clock (*Ryr2*, *Slc8a1*/NCX1), consistent with some of the studies above. Interestingly, there was evidence of activation of the immune system, for example an upregulation of the transcripts responsible for the major histocompatibility complex class 2 (Fig. 6F). This may be important as discussed below.

There have been two other recent reviews on age-dependent sinus node dysfunction (Monfredi & Boyett, 2015; Peters *et al.*, 2020).

**9. Regulation of the HCN channels in disease - heart failure**

Although chronic heart failure patients can exhibit heart rates greater than 70 beats/min (example shown in Fig. 7A) (Crespo-Leiro *et al.*, 2016), because of an activation of the sympathetic nervous system to support the failing heart (Hasking *et al.*, 1986; Parati & Esler, 2012), there is evidence of underlying sinus node dysfunction in heart failure patients: (i) Jose *et al.* (Jose & Taylor, 1969) showed that there is a decrease in the *intrinsic heart rate* (measured after complete autonomic blockade), which worsened with the severity of heart failure (Fig. 7B); (ii) Sanders *et al.* (Sanders *et al.*, 2004) showed functional sinus node remodelling, with a prolonged intrinsic sinus node cycle length, corrected sinus node recovery time and sinoatrial conduction time; (iii) in the CARSIMA study of post-myocardial infarction heart failure patients (Bloch Thomsen *et al.*, 2010), sinus bradycardia was observed in 6.7% of the patients over a two year period (Fig. 7C); and (iv) ~70% of heart failure patients (Roche *et al.*, 2001) show chronotropic incompetence, the inability of the heart rate to appropriately rise with exertion perhaps as a result of sinus node dysfunction (Sanders *et al.*, 2004;

Brubaker & Kitzman, 2011). Evidence suggests that the sinus node dysfunction impacts heart failure patients: in the CARISMA study, sinus bradycardia was a more significant predictor of mortality than non-sustained ventricular tachycardia (Fig. 7D); there is a J-shaped relationship between mortality and heart rate in chronic heart disease and, although there is an obvious association of mortality with a higher heart rate, there is also an association with a lower heart rate (Kolloch *et al.*, 2008; Parodi *et al.*, 2010); and a significant proportion of heart failure patients (27.6% – 62%) are profoundly bradycardic at the point of sudden death (Luu *et al.*, 1989; Schoeller *et al.*, 1993; Stevenson *et al.*, 1993; Faggiano *et al.*, 2001).

Could bradyarrhythmias cause heart failure? Iwataki *et al*. (Iwataki *et al.*, 2015) compared 39 patients with a mean heart rate of 84±17 beats/min and heart failure, which they attributed to left ventricular pump failure, with 24 patients with a mean heart rate of 39±5 beats/min and heart failure, which they attributed to the bradyarrhythmia; in both groups there was a decrease in cardiac output and increase in left ventricle filling pressure (the latter was only modest in the bradyarrthmic patients). However, the study of Iwataki *et al*. (Iwataki *et al.*, 2015) is inconclusive: although Iwataki *et al*. (Iwataki *et al.*, 2015) attributed the heart failure in the bradycardic patients to the bradyarrhythmia, it is also possible that the bradyarrhythmia is a consequence of the heart failure. Penton *et al*. (Penton *et al.*, 1956) studied 251 AV (i.e. heart) block patients and observed congestive heart failure in 40% of the patients. They reported that the congestive heart failure could “precede, accompany, or follow the onset of complete AV block”; in patients in which the congestive heart failure followed the onset of complete AV block, the congestive heart failure could have been caused by the bradyarrhythmia. Alboni *et al.* (Alboni *et al.*, 1999) pointed out that improvement in symptoms of heart failure, with or without the addition of digitalis, has been reported after pacemaker implantation in patients with sick sinus syndrome, and pacing is commonly considered an effective treatment for patients with sinus bradycardia and heart failure. In the randomised THEOPACE trial involving 107 patients with symptomatic sinus bradycardia (sinus rate <50 beats/min) from the same authors, an increase in heart rate, induced by DDD pacing or oral theophylline, significantly reduced the incidence of overt heart failure (Alboni *et al.*, 1997; Alboni *et al.*, 1999; Alboni *et al.*, 2001); the authors concluded that sinus bradycardia appears to play a role in the genesis of heart failure. Case reports support the concept that bradycardia can lead to new symptoms of heart failure and

decompensation of pre-existing heart failure (Berczeller, 1994; Ntalianis & Nanas, 2006; Senga *et al.*, 2007; Caliskan *et al.*, 2010; Aoun & Tabbah, 2016; Nagatomo *et al.*, 2020), but the anecdotal evidence of case reports has to be treated cautiously. Two studies relating to HCN4 and $I_f$ will be highlighted: Milano *et al*. (Milano *et al.*, 2014) showed that, in four families, HCN4 mutations linked to bradycardia are associated with left ventricular noncompaction cardiomyopathy, whereas Romero-León *et al*. (Romero-Leon *et al.*, 2016) reported that the blocker of HCN4 and $I_f$, ivabradine, induced heart failure in a patient receiving antiviral therapy, and when ivabradine was withdrawn symptoms disappeared. Once again, the publication of Romero-León *et al*. (Romero-Leon *et al.*, 2016) is a case report and as such has to be treated cautiously. In conclusion, there is clinical evidence that bradyarrhythmias, in conjunction with pre-existing disease, may contribute to the symptoms and signs of heart failure. However, the authors know that this concept is controversial and not widely accepted in the clinical community; clearly, there is a need for further clinical studies.

Dysfunction of the sinus node has been observed in a rabbit model of pressure and volume overload-induced heart failure, a dog ventricular tachypacing-induced heart failure model, a rat model of volume overload-induced heart failure, and a mouse model of pressure overload-induced heart failure (Opthof *et al.*, 2000; Verkerk *et al.*, 2003; Zicha *et al.*, 2005; Du *et al.*, 2016; Yanni *et al.*, 2020). These studies have shown that the heart rate measured *in vivo* in the conscious animal can be slowed, the intrinsic heart rate measured *in vivo* after complete autonomic blockade is slowed, the intrinsic pacemaker activity of the sinus node *in vitro* is slowed, HCN4 mRNA and protein is downregulated, and $I_f$ is downregulated. What is the underlying mechanism? Yanni *et al*. (Yanni *et al.*, 2020) screened 754 microRNAs in the sinus node using qPCR and observed significant changes in 44 of them (some upregulated and others downregulated) in the mouse model of pressure overload-induced heart failure. miR-370-3p and miR-139-3p were upregulated (miR-370-3p was shown to be also upregulated in the human sinus node in heart failure) and they were identified by Ingenuity Pathway Analysis software as potentially targeting HCN4 (Yanni *et al.*, 2020). RNA22 software predicted that *Hcn4* mRNA has multiple binding sites for both microRNAs and luciferase reporter gene assays confirmed that the two microRNAs can bind to and downregulate *Hcn4* mRNA (Yanni *et al.*, 2020). Exogenous expression of the two microRNAs in the isolated sinus node caused a downregulation of HCN4 protein and a decrease in the intrinsic pacemaker activity

(Yanni *et al.*, 2020). *In vivo*, silencing of miR-370-3p by an intraperitoneal injection of a suitable anti-microRNA prevented the upregulation of miR-370-3p, prevented the downregulation of HCN4 mRNA and protein and blunted the decrease in heart rate (Fig. 8A) (Yanni *et al.*, 2020). Fascinatingly, Yanni *et al.* (Yanni *et al.*, 2020) showed that the anti-miR-370-3p reduced cardiac hypertrophy and most importantly reduced mortality. This is consistent with the decrease in heart rate worsening heart failure and mortality - the anti-microRNA by blunting the decrease in heart rate ultimately blunts mortality. There are case reports of heart failure patients in whom heart failure symptoms were stabilised, reduced or even reversed by cessation of ivabradine treatment (Romero-Leon *et al.*, 2016) or implantation of a pacemaker (Senga *et al.*, 2007; Caliskan *et al.*, 2010; Nagatomo *et al.*, 2020). What upstream pathway is responsible for the microRNA changes? As already mentioned, there is well known to be an activation the sympathetic nervous system to support the failing heart (Hasking *et al.*, 1986; Parati & Esler, 2012). Du *et al*. (Du *et al.*, 2016) have reported that treatment of a rat model of volume overload-induced heart failure with the beta-blocker, bisoprolol, prevented the heart failure-induced downregulation of *Hcn4* mRNA, decrease in the intrinsic heart rate, and the increase in the sinus node recovery time (Fig. 8B). This suggests that the sinus node remodelling in heart failure may be ultimately due to the hyperadrenergic state. This deserves further investigation, but of course beta-blockers are already a mainstream therapy used to treat heart failure.

Fig. 8C attempts to present a synthesis of this information. The inner loop in black is well known and accepted: during heart failure there is activation of the sympathetic nervous system, which results is an inappropriately fast heart rate; this stresses the heart and ultimately worsens the condition of heart failure. The outer loop in red is more speculative and summarises the information presented here. The activation of the sympathetic nervous system in heart failure or perhaps the activation of the immune system in heart failure (see below) causes a remodelling of the sinus node: there is an upregulation of some microRNAs targeting *Hcn4* resulting in a downregulation of HCN4 and $I_f$ and consequently sinus node dysfunction. It is well known that heart failure patients suffer chronotropic incompetence (an inability of the heart rate to increase sufficiently on exercise) (Brubaker & Kitzman, 2011) and this could be the result of the sinus node dysfunction. The resulting relative sinus bradycardia could worsen the condition of heart failure.

A common cause of heart failure is myocardial infarction and a rat model of myocardial infarction-induced heart failure showed sinus node dysfunction: the intrinsic heart rate was reduced and the corrected sinus node recovery time increased (Yanni *et al.*, 2011). qPCR showed an increase in the expression of ERG, KvLQT1, Kir2.4, TASK1, TWIK1, TWIK2, calsequestrin 2 and A1 adenosine receptor transcripts in the sinus node that could explain the slowing of the intrinsic heart rate (Yanni *et al.*, 2011). In this rat model, HCN channels were not downregulated: *Hcn2* and *Hcn4* were upregulated in the sinus node in heart failure (Yanni *et al.*, 2011). Therefore, as with ageing, the remodelling of the sinus node in heart failure can take different forms.

**10. Regulation of the HCN channels in disease – pulmonary hypertension**

Pulmonary hypertension (pulmonary artery pressure >25 mmHg) is a disease characterised by raised pulmonary vascular resistance. It has a poor prognosis and typically results in progressive right ventricular failure and death. The incidence of arrhythmias in patients with pulmonary hypertension is high (Temple *et al.*, 2016). For example, in patients with pulmonary hypertension, sinus tachycardia, sinus bradycardia and first-degree AV block occurred in 70% of the patients, whereas ventricular arrhythmias were rare (Kanemoto & Sasamoto, 1979; Rajdev *et al.*, 2012). In a rat model of monocrotaline-induced pulmonary hypertension, there was a decrease of the intrinsic heart rate (measured in the isolated sinus node) as well as the normal heart rate, evidence of sinus node dysfunction (Yamanushi *et al.*, 2010). qPCR and immunohistochemistry showed a remodelling of ion channel, $Ca^{2+}$-handling and fibrosis genes in the sinus node which was likely to be responsible for the sinus node dysfunction (Yamanushi *et al.*, 2010). This included a downregulation of *Hcn1* and *Hcn4* (Fig. 9A,B) (Yamanushi *et al.*, 2010).

**11. Regulation of the HCN channels in disease – diabetes mellitus**

The major cause of morbidity and mortality in diabetic patients are cardiovascular complications. Bradyarrhythmias (Grimm *et al.*, 1990) and AV block (Rubler *et al.*, 1975) are significant problems in diabetes mellitus. Diabetic patients are more likely to need implantation of an electronic pacemaker. Data from 688 elderly patients (79% aged over 65 years) who had undergone electronic pacemaker implantation were compared to data from a control group (Lear *et al.*, 1996): the relative risk of needing an electronic pacemaker was 1.34 for the elderly diabetic patients. In a large study, 416,247 patients with type 2 diabetes mellitus from the Swedish National Diabetes Registry were

compared with >2,000,000 matched control subjects – for diabetic patients, the relative risk of needing an electronic pacemaker was 1.65 (Rautio *et al.*, 2018; Rautio *et al.*, 2020).

Dobrzynski and Howarth have studied nodal function in streptozotocin-induced type 1 diabetic rats: there is a decrease in the heart rate *in vivo* (Howarth *et al.*, 2005a; Howarth *et al.*, 2005b) and *in vitro* there is a decrease of the beating rate of the isolated (and denervated) sinus node preparation (Zhang *et al.*, 2019). Huang *et al*. (Huang *et al.*, 2016) have similarly reported a decrease in heart rate *in vivo* and beating rate *in vitro*, but in addition a lengthened sinoatrial conduction time and rate-corrected maximal sinus node recovery time *in vivo* in the streptozotocin-induced type 1 diabetic rat. Ferdous *et al*. (Ferdous *et al.*, 2016) reported changes in various transcripts in the sinus node in streptozotocin-induced type 1 diabetic rat, but no change in *Hcn4* mRNA. However, there is doubt about this conclusion, because Ferdous *et al*. (Ferdous *et al.*, 2016) reported *Hcn4* mRNA to be higher in the right atrial muscle than in the sinus node of the control animals and this contradicts many previous reports, e.g. Tellez *et al.* (Tellez *et al.*, 2006). Furthermore, Huang *et al*. (Huang *et al.*, 2016) reported a downregulation on HCN2 and HCN4 mRNA and protein in the sinus node of the streptozotocin-induced type 1 diabetic rat; they also reported that blockade of HCN channels by 3 μM ivabradine prolonged the spontaneous cycle length by 18% in Langendorff hearts from diabetic rats and 26% from control rats, consistent with a downregulation of $I_f$ in the diabetic rats. This is likely to be correct, because Zhang *et al*. (Zhang *et al.*, 2019) - from the same team as the paper by Ferdous *et al*. (Ferdous *et al.*, 2016) - showed using western blot that HCN4 protein is downregulated in the sinus node of streptozotocin-induced type 1 diabetic rats. Zhang et al. (Zhang *et al.*, 2019) also showed a downregulation of other key proteins involved in pacemaking (Cav1.3, Cav3.1, Cx45 and NCX1).

**12. Regulation of the HCN channels in disease – atrial fibrillation**

Sinus node dysfunction is frequently associated with AF resulting in the “tachy-brady syndrome” (Semelka *et al.*, 2013). AF is responsible for the tachycardia and when in sinus rhythm the patient is bradycardic. Sick sinus syndrome after termination of AF can cause syncope and require pacemaker implantation (Yeh *et al.*, 2009). In the dog, Elvan *et al.* (Elvan *et al.*, 1996) reported that after 2-6 weeks of atrial tachypacing to induce AF, there was sinus node dysfunction: the maximal and intrinsic heart rates were decreased and the corrected sinus node recovery time was prolonged. Yeh *et al*.

(Yeh *et al.*, 2009) studied sinus node dysfunction in the dog resulting from seven day atrial tachypacing. In the sinus node, *Hcn2* and *Hcn4* mRNAs as determined by qPCR were downregulated by >50% (Fig. 10A) and mRNA for minK (an accessory subunit for KvLQT1 responsible for the slow delayed rectifier $K^+$ current, $I_{K,s}$) was downregulated by ~42%. Patch clamp experiments showed a downregulation of the corresponding currents, $I_f$ and $I_{K,s}$, respectively (Yeh *et al.*, 2009); data for $I_f$ are shown in Fig. 10B. Yeh *et al*. (Yeh *et al.*, 2009) concluded that the downregulation of $I_f$ in particular may be responsible for the sinus node dysfunction.

**13. Other bradycardias – other HCN problems?**

A series of publications report bradycardia and bradyarrhythmias in COVID-19 patients (Amaratunga *et al.*, 2020; Babapoor-Farrokhran *et al.*, 2020; Beyls *et al.*, 2020; Bhatla *et al.*, 2020; Capoferri *et al.*, 2020; Cimino *et al.*, 2020; Hiraiwa *et al.*, 2020; Hu *et al.*, 2020; Ikeuchi *et al.*, 2020; Manolis *et al.*, 2020; Peigh *et al.*, 2020). It has been reported: relative bradycardia is a frequent clinical feature of COVID-19, occurring in 56% of febrile hospitalised patients (Capoferri *et al.*, 2020); about a third of the patients with severe illness develop sinus bradycardia (Hu *et al.*, 2020); and one of the most common arrhythmias in relation to COVID-19 is sinus bradycardia (Babapoor-Farrokhran *et al.*, 2020). There is a high mortality of patients with chronic kidney disease undergoing haemodialysis and 25% of deaths are the result of sudden cardiac death (Wong *et al.*, 2015a). In 50 of these patients, bradycardia was recorded in 30%, sinus arrest in 28% and second-degree AV block in 8%; 16% of the patients died of sudden cardiac death and in each case there was severe bradycardia with asystole (Wong *et al.*, 2015b). Anorexia nervosa is an eating disorder with a significant risk for sudden death (5-20%) due to severe cardiovascular complications. Anorexia nervosa is associated with bradycardia - in a study of 20 female patients, ~70% had a heart rate of <50 beats/min and the mean lowest heart rate was 44 beats/min (Yahalom *et al.*, 2013). There was a significant correlation between heart rate and body mass index (Yahalom *et al.*, 2013). Similar findings have been reported by others (Kollai *et al.*, 1994; Vanderdonckt *et al.*, 2001; Portilla, 2011). Although the cause of sinus bradycardia in these cases is not known, a transcriptional remodelling of the sinus node, including of HCN channels, perhaps as a result of inflammation, must be one possible cause. In the case of COVID-19 there is some evidence for this: miR-486-3p is highly expressed in the human sinus node and inhibits HCN4 expression (Petkova *et al.*, 2020; Aminu *et al.*, 2021) and circulating miR-486-3p

is upregulated in COVID-19 patients (Tang *et al.*, 2020); sinus bradycardia in COVID-19 patients has also been attributed to inflammatory damage of sinus node cells (Amaratunga *et al.*, 2020).

**14. AV node and HCN channels in health and disease**

Most studies on the nodes in health and disease focus on the sinus node and this review reflects this bias. However, there are studies of the HCN channels in the AV node in health and disease. HCN channels are considered to be pacemaking ion channels. The primary function of the AV node is to conduct the action potential from the atria to the ventricles, although it is also a back-up pacemaker in case of failure of the sinus node (Dobrzynski *et al.*, 2003). As in the case of the sinus node, the HCN channels of the AV node are responsible for $I_f$, which is involved in AV node pacemaking (Liu *et al.*, 2008; Marger *et al.*, 2011; Mesirca *et al.*, 2014). However, there is increasing evidence that $I_f$ is involved in AV node conduction:

(i) HCN4 mutations have been associated with AV block in humans (Zhou *et al.*, 2014a).

(ii) Baruscotti *et al*. (Baruscotti *et al.*, 2011) demonstrated that inducible cardiac-specific knockout of HCN4 in mice is lethal because of the development of complete AV block. Similarly, Mesirca *et al.* (Mesirca *et al.*, 2014) showed that inducible cardiac-specific silencing of f-channel conductance after expression of dominant-negative non-conducting HCN4 in mice resulted in a high incidence of types 1 and 2 second-degree AV block, as well as a reduction of automaticity of AV node myocytes.

(iii) The $I_f$ blocking agent, zatebradine, has been shown to increase the A-H interval, AV node effective refractory period, and Wenckebach cycle length in humans (Chiamvimonvat *et al.*, 1998); similarly, in the rat, $I_f$ block by ivabradine prolongs the A-H interval, AV node effective refractory period, and Wenckebach cycle length (Saeed *et al.*, 2018).

(iv) Verrier *et al.* (Verrier *et al.*, 2014) reported that ivabradine, at clinically safe concentrations, reduces ventricular rate during AF in the guinea-pig by slowing AV node conduction. Consistent with this, there are various reports that ivabradine improves ventricular rate control in patients with AF (Caminiti *et al.*, 2016; Wongcharoen *et al.*, 2016; Fontenla *et al.*, 2017).

We suggest that downregulation of $I_f$ may slow AV node conduction by decreasing cell excitability (Li *et al.*, 2014). In this respect, previous work on the hippocampus by Maccaferri *et al*. (Maccaferri *et al.*, 1993) has shown that $I_h$ ($I_f$) positively regulates neuronal firing by providing a

depolarization reserve at resting potentials from and negative to -50 mV. A similar mechanism may be operating in the AV node to facilitate action potential conduction. Proenza and Yellen (Proenza & Yellen, 2006) reported that, on hyperpolarization, HCN channels may carry an instantaneous, time-independent current as well as a time-dependent current. Perhaps as an example of this, Fig. 3 of Kozasa *et al*. (Kozasa *et al.*, 2018) shows comparable changes of both time-independent and time-dependent currents as the HCN4 expression level was varied. Such an instantaneous, time-independent current, by mimicking a background inward current, could facilitate AV node conduction by $I_f$.

***Athletic training.*** AV node dysfunction is a common occurrence in athletes, manifesting as a longer PR interval (first-degree AV block, i.e. slowed AV node conduction), a prolonged Wenckebach cycle length (minimum cycle length at which the AV node fails to conduct in a 1:1 manner) and second-degree AV block (intermittent block of conduction through the AV node) (Viitasalo *et al.*, 1982; Stein *et al.*, 2002; Crouse *et al.*, 2009). AV block has been correlated to the intensity and length of the training period in athletes (Hoogsteen *et al.*, 2004). The prevalence of first- and second-degree AV block in veteran endurance athletes has been reported to be 28–45% and 15–40% (Talan *et al.*, 1982) in contrast to 0.60% and 0.03% in the general population (Hiss & Lamb, 1962). The AV block likely contributes to the increased incidence of electronic pacemaker implantation in veteran athletes (Hood & Northcote, 1999; Luthi *et al.*, 2008). Like the resting bradycardia in athletes, the AV block is attributed to high vagal tone (D'Souza *et al.*, 2019). However, Stein *et al.* (Stein *et al.*, 2002) reported that the Wenckebach cycle length and anterograde AV node effective refractory period are prolonged in human athletes, regardless of the presence of autonomic blockade. Similarly, Yamaya *et al.* (Yamaya *et al.*, 1997) reported that slower AV conduction, prolonged Wenckebach conduction and higher refractoriness in horses with AV block (*versus* horses without AV block) was maintained under complete autonomic block. Mesirca *et al.* (Mesirca *et al.*, 2021) reported that, as compared to sedentary animals, exercise-trained horses presented with a prolonged PR interval and exercise-trained mice also presented with a prolonged PR interval (*in vivo* and *ex vivo*) as well as a prolonged Wenckebach cycle length and AV node refractory period; the prolongation of the PR interval persisted after complete autonomic blockade in both species. In the exercise-trained mice, there was a downregulation of Cav1.2 and HCN4 mRNA (qPCR) and protein

(immunohistochemistry and western blot) in the AV node and there was a concomitant decrease in the density of $I_{Ca,L}$ and $I_f$ (patch clamp) (Mesirca *et al.*, 2021). There was a similar downregulation of Cav1.2 and HCN4 proteins in the exercise-trained horses. In the exercise-trained mice, a downregulation of other ion channel transcripts was also observed (Mesirca *et al.*, 2021). microRNA profiling revealed an upregulation of miR-211-5p and miR-432 in the AV node of the exercise-trained mice and evidence of a similar upregulation in the exercise-trained horses, and *in vitro* studies showed the two microRNAs to be direct regulators of Cav1.2 and HCN4 (Mesirca *et al.*, 2021). In the mouse, *in vivo* suppression of the microRNAs reversed training-induced PR prolongation and ion channel remodelling (Mesirca *et al.*, 2021).

***Circadian rhythm.*** In the human and the mouse, there is a circadian rhythm in the PR interval, which is longer (i.e. AV node conduction is slower) during the sleep period (Dilaveris *et al.*, 2001; Black *et al.*, 2018). Without direct evidence, the circadian rhythm has again been attributed to the autonomic nervous system (principally, high vagal tone during sleep) (Verrier & Josephson, 2009). However, as in the case of the sinus node, qPCR has shown a circadian rhythm in the expression of circadian clock and ion channel transcripts in the AV node of young male C56BL/6J mice (Sunil Jit Logantha and M.R. Boyett); see D'Souza *et al*.(D'Souza *et al.*, 2021) for details of methods. The circadian rhythm in *Hcn4* is shown in Fig. 11A and it can be seen to be similar in phase with the circadian rhythm in *Hcn4* in the sinus node (Fig. 4B).

***Heart failure and pulmonary hypertension.*** Heart failure is not only associated with sinus node dysfunction, evidence suggests that it is also associated with AV node dysfunction. Among patients with heart failure, first-degree AV block is present in 15-51% of patients as compared to a prevalence of ~4% in the general population (Nikolaidou *et al.*, 2016). In heart failure patients, first-degree AV block is associated with an increased risk of mortality and heart failure hospitalisation, and optimisation of AV delay in patients by means of cardiac resynchronisation therapy is an important therapy (Nikolaidou *et al.*, 2016). AV node dysfunction has been observed in animal models of heart failure. Muir-Nisbet *et al*. (Muir-Nisbet, 2008) observed an increase in the PQ interval (equivalent to first-degree AV block) in a rabbit model of ischaemic cardiomyopathy (resulting from left ventricular myocardial infarction), which they attributed to increased fibrosis and gap junction (Cx43 and Cx40) remodelling. We have observed a prolongation of the PR interval (equivalent to

first-degree AV block) in a rabbit model of pressure and volume overload-induced heart failure caused by rupture of the aortic valve and banding of the abdominal aorta (Nikolaidou *et al.*, 2015). An increase in the PR interval results from either slower conduction through the AV node or an increase in the length of the conduction pathway. In the rabbit heart failure model, there was a downregulation of HCN1 as measured by qPCR in the AV junction that could result in a slowing of conduction (Nikolaidou *et al.*, 2015). However, there was also a downregulation of Cav1.3, Cx40 and Cx43 mRNAs, which again could result in a slowing of conduction, as well as an enlargement of the AV junction (and the rest of the heart) as measured by micro-CT, which could result in an increase in the length of the conduction pathway (Nikolaidou *et al.*, 2015). In the work of Muir-Nisbet *et al*. (Muir-Nisbet, 2008) and Nikolaidou *et al.* (Nikolaidou *et al.*, 2015), the initial insult was to the left side of the heart. We have also studied the rat model of monocrotaline-induced pulmonary hypertension (Temple *et al.*, 2016). There is evidence of AV node dysfunction in pulmonary hypertension with a 14% incidence of first-degree heart block (Bossone *et al.*, 2002) compared with 2.1% in the general population (Aro *et al.*, 2014), a mean PR interval of ~180 ms (Bossone *et al.*, 2002) compared with ~160 ms for men and ~153 ms for women in the general population (Aro *et al.*, 2014), and 2% of patients requiring a pacemaker for high-degree heart block on initial screening of pulmonary hypertension patients (Bossone *et al.*, 2002). Sleep apnoea causes pulmonary hypertension (Laks *et al.*, 1995) and is associated with heart block in ≤10% of patients with obstructive sleep apnoea (Koehler *et al.*, 1998). In the rat model of monocrotaline-induced pulmonary hypertension, Temple *et al*. (Temple *et al.*, 2016) showed that there was a 50% incidence of heart block in isolated AV node preparations and a widespread downregulation of ion channel and related genes in the AV node. For example, there was a >50% downregulation of *Hcn1/2/4* and Cav1.2 and Cav1.3 mRNAs (Fig. 11B); biophysically-detailed computer modelling by Zhang and colleagues predicted that if the changes in mRNA are translated into protein and function they would result in heart block (Temple *et al.*, 2016).

Finally, in the mouse model of pressure overload-induced heart failure caused by banding of the aorta, there is a prolongation of the PR interval (Yanni *et al.*, 2020) and in this model we (Wilson and Boyett) have used RNAseq to study the transcriptome of the AV node. Biopsies from nine heart failure and nine sham-operated control mice (young male C57BL/6J mice) each divided into three

groups were used; see Yanni *et al.* (Yanni *et al.*, 2020) and Wang *et al*. (Wang *et al.*, 2021) for details of methods. There were widespread changes in the transcriptome including a downregulation of $Ca^{2+}$ and $K^{+}$ channel transcripts, although there were no changes in *Hcn* transcripts. The data also showed evidence of an activation of the immune system and either an infiltration of the AV node by different classes of inflammatory cells or a proliferation of the cells in the AV node. This is of interest because heart failure is characterised by inflammation (Martini *et al.*, 2019) and inflammation can cause heart failure: myocardial inflammation and T-cells can lead to lethal acute or chronic heart failure (Blanton *et al.*, 2019; Strassheim *et al.*, 2019). In the same mouse model of pressure overload-induced heart failure, knockout of the class II major histocompatibility complex molecule or knockout of *Tcra* (T-cell receptor alpha locus) resulted in the nearly complete inhibition of left ventricular hypertrophy, and, in the case of knockout of *Tcra*, maladaptive changes in gene expression were prevented (Laroumanie *et al.*, 2014; Blanton *et al.*, 2019). The possibility that the remodelling of the nodes in heart failure is caused by inflammation is incorporated in Fig. 8C.

**15. Biopacemaking**

Currently, the only treatment for nodal dysfunction is the implantation of an electronic pacemaker, a treatment that has been in use for ~62 years (Aquilina, 2006). However, implanted electronic pacemakers have significant problems, for example, a limited battery life (Morris & Boyett, 2009). This has sparked the development of a 'biopacemaker' (an artificial biological pacemaker) as a replacement for the electronic pacemaker - authoritative reviews on biopacemakers have already been published (Rosen *et al.*, 2007; Morris & Boyett, 2009) and the work will not be reviewed in detail here. However, it is relevant to this review that many efforts to generate a biopacemaker have focussed on the HCN channels (Rosen *et al.*, 2007). Most studies have focussed on a *de novo* pacemaker in the working myocardium (Rosen *et al.*, 2007). However, this may not be satisfactory – it is known that electronic pacemaking in the right ventricle can lead to pacing-induced cardiomyopathy because it does not replicate the normal activation sequence of the heart (Merchant & Mittal, 2020). It is, therefore, important to retain the normal activation sequence of the heart. In addition, most studies attempting to develop a biopacemaker focus on changing one or two genes, whereas it is known that there are 100s of differences in gene expression between the sinus node and atrial muscle (Linscheid *et al.*, 2019). We have argued that it may be better to 'repair' the existing

nodal tissues using gene therapy if the nature of the problem is known (Morris *et al.*, 2013; Choudhury *et al.*, 2018). Sinus node tissue is extensive in the right atrium and it is the superior tissue close to the superior vena cava which is the dominant pacemaker (showing the fastest pacemaking) – see also Brennan *et al.* (Brennan *et al.*, 2020). We have worked with the isolated inferior part of the sinus node and shown that pacemaking can be accelerated by transducing the tissue with a HCN chimeric channel, HCN212 (Morris *et al.*, 2013) and TBX18 (Choudhury *et al.*, 2018). An important problem yet to be overcome is how nodal tissue can be selectively transduced, i.e. how one of the nodes can be genetically modified without affecting any other tissue in the heart or body (Stuart, 2021).

**16. Purkinje fibres – an afterthought**

This review focusses on the role of HCN channels in the sinus and AV nodes in health and disease, but what about the final part of the cardiac conduction system, the Purkinje fibres? Paradoxically, although $I_f$ was first recorded in Purkinje fibres (Noble & Tsien, 1968), less is known about the current in Purkinje fibres. It is attributed to HCN channels and is thought to play a role in pacemaking (Boyden *et al.*, 2010; Dobrzynski *et al.*, 2013). Purkinje fibres are involved in a variety of arrhythmias. For example, Purkinje fibre dysfunction can result in bundle branch block, the incidence of which is higher in athletes (incomplete right bundle branch block), the elderly and heart failure patients (Dobrzynski *et al.*, 2013; Scharhag *et al.*, 2013). However, there is no evidence that $I_f$ is involved in Purkinje fibre dysfunction, although *Hcn4* mRNA is downregulated in left ventricular Purkinje fibres in a rabbit model of pressure and volume overload-induced heart failure (Logantha *et al.*, 2021).

**17. Conclusions**

There are changes in the base line (i.e. long term) heart rate set by the sinus node and also AV node conduction in many different physiological and pathophysiological conditions. Under physiological conditions, the changes adapt the heart to the changing need for cardiac output. However, under pathophysiological conditions the changes can be maladaptive. Previously, a lack of information and understanding of the two nodes meant that changes were attributed to high vagal tone or fibrosis, because at the time these were the only factors thought to affect the nodes. However, as the knowledge and understanding of the nodes increases further factors that could be involved have come to light and deserve investigation. This review has focussed on a role for the important

pacemaker HCN ion channels and it has been shown that transcriptional control of the channels and consequently $I_f$ density is occurring in many different physiological and pathophysiological circumstances (Fig. 12A) through a variety of mechanisms (Fig. 12B) and could either be responsible for or contributing to a change in heart rate and AV node conduction. It is important to stress that, although this review focusses on the HCN channels, it does not mean that other factors such as the $Ca^{2+}$ clock are not involved. *Hcn4* mutations are associated with sinus node dysfunction and other diseases and the *Genomics England* website states that *Hcn4* should be analysed when investigating 'Progressive cardiac conduction disease' (which includes sick sinus syndrome) and 'Cardiomyopathies - including childhood onset' (https://panelapp.genomicsengland.co.uk/panels/entities/HCN4).

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## Figure legends

**Fig. 1. Expression of HCN4 protein in the sinus node. A**, micro-CT image of a virtual section of the right atrium (RA) of the rabbit heart. The section is cut perpendicular to the crista terminalis (CT) and runs through the sinus node (SAN). **B**, volume rendering based on micro-CT data of the rear wall of the right atrium of the rabbit heart showing the superior and inferior venae cavae (SVC and IVC), sinus node and crista terminalis (PM, pectinate muscles). **C**, segmented 3D surface based on micro-CT data of the sinus node of the rabbit heart showing the sinus node (SAN; yellow) and sinus node artery (SNA; red). **D**, volume rendering based on micro-CT data of the rear wall of the right atrium of the human heart showing the superior and inferior venae cavae (SCV and ICV), sinus node (SN; outlined by a dashed yellow line) and crista terminalis. PcM, pectinate muscles. **E**, section cut perpendicular to the crista terminalis through the intercaval region of the rat heart immunolabelled for Cx43 (red signal) and HCN4 (green signal). RSARB, right sinoatrial ring bundle. A-C from Stephenson *et al.* (Stephenson *et al.*, 2012), D from Stephenson *et al.* (Stephenson *et al.*, 2017) and E from Boyett (Boyett, 2009).

**Fig. 2. Resting bradycardia in the athlete and the involvement of HCN4 and $I_f$. A**, distribution of resting heart rates in elite athletes and a normal population. Red bars, histogram of resting heart rate of 142 elite cyclists and rowers. Grey bars, histogram of resting heart rate of 3,061 20- to 39-year-old female subjects. From D'Souza *et al.* (D'Souza *et al.*, 2015). **B**, mean hourly heart rate (± SEM) of 20 athletes and 20 control subjects over 24 h; for visual purposes, the data are repeated for a second 24 h period. From Northcote *et al*. (Northcote *et al.*, 1989). **C**, density of $I_f$ is reduced in trained mice. Left, representative $I_f$ traces, normalised to cell capacitance, during steps to -55 to -135 mV from a holding potential of -35 mV from single sinus node cells isolated from sedentary and exercise trained mice. Right, mean (± SEM) $I_f$ current-voltage relationships from sedentary and exercise trained mice. **D**, antimiR abolishes the exercise training-induced upregulation of miR-423-5p in the sinus node (measured by qPCR; left panel), downregulation of HCN4 protein in the sinus node (measured by western blot; central panel), and decrease in heart rate measured *in vivo* (right panel). Mean ± SEM data for control (sedentary) mice, exercise trained mice, and antimiR treated exercise trained mice shown. In all figures, asterisks indicate significant differences.

**Fig. 3. Molecular pathways responsible for the resting bradycardia in the athlete. A**, schematic diagram of potential events during exercise training. **B** and **C**, effect of exercise training on miR-423-5p and *Hcn4* in the sinus node of wild-type and *Girk4* (also known as Kir3.4 and *Kcnj5*) knockout mice. Black bars, data from control sedentary mice; red bars, data from exercise trained mice. Means ± SEM shown. **D**, representative HCN4 western blot (upper panel) with corresponding stain-free total protein blot used for quantification (lower panel) in sinus node biopsies isolated from sedentary and trained wild-type or *Girk4*$^{-/-}$ mice. **E**, HCN4 protein expression determined by western blot from data given in D. Black bars, data from control sedentary mice; red bars, data from exercise trained mice. Modified from Bidaud *et al.* (Bidaud *et al.*, 2020b). In B, C and E, means ± SEM shown.

**Fig. 4. Circadian rhythm in the sinus node and the involvement of HCN4 and $I_f$. A,** schematic diagram of the circadian clock (left) and circadian rhythm in two example clock transcripts (right). On the right, mean ± SEM transcript abundance shown at six zeitgeber time (ZT) time points over 24 h; the permutation-based P value from JTK Cycle for significance of a day-night rhythm is given; the data have been fitted with a sine wave by a least squares fitting method and the $R^2$ value is also given. **B**, families of recordings of $I_f$ made from sinus node cells isolated at ZT 6 and ZT 12 (left) and mean (± SEM) current-voltage relationships for $I_f$ recorded from sinus node cells isolated at ZT 6 and ZT 12 (right). ZT 0 is the start of the 12 h light period and ZT 12 is the start of the 12 h dark period. **C** and **D**, HCN4 protein expression from western blot and density of $I_f$ at -120 mV at ZT 6 and ZT 12. **E,** *in vivo* heart rate of wild-type mice measured at ZT 0 (white bars) and ZT 12 (black bars) before and after the administration of ivabradine. In all relevant panels, means ± SEM shown. Modified from D'Souza *et al*. (D'Souza *et al.*, 2021) and Wang *et al*. (Wang *et al.*, 2021).

**Fig. 5. Heart rate during pregnancy and postnatal development and the involvement of HCN4 and $I_f$. A**, changes in heart rate during pregnancy in the human. Modified from Hall *et al*. (Hall *et al.*, 2011). **B**, changes in mean (± SEM) heart rate whilst awake and during quiet sleep from the neonate to the young adult in the human (n=8-18). Based on data from Finley and Nugent (Finley & Nugent,

1995). **C**, families of recordings of $I_f$ made from sinus node cells isolated from non-pregnant and pregnant mice (left) and mean (± SEM) current-voltage relationships for $I_f$ in the two sets of mice (right) (n=26 cells for both non-pregnant and pregnant mice). Modified from El Khoury *et al*. (El Khoury *et al.*, 2013). **D**, mean (± SEM) abundance of *Hcn1* and *Hcn4* transcripts in the sinus node, right atrium and left ventricle of neonatal and adult rabbits (n=8 for both neonate and adult). From Abd Allah *et al.* (Abd Allah *et al.*, 2011).

**Fig. 6. Age-dependent sinus node dysfunction and the involvement of HCN4 and $I_f$. A** and **B**, relationship between the normal heart rate (A) and the intrinsic heart rate measured after complete autonomic blockade (B) and age amongst 56 human subjects. Modified from Kuga *et al.* (Kuga *et al.*, 1993). The dashed lines in B correspond to ± 1 standard deviation. The shaded area corresponds to the 95% confidence limit for the intrinsic heart rate from Jose and Collison (Jose & Collison, 1970). **C**, relationship between sick sinus syndrome and age in 90 female (left hand bars) and male (right hand bars) patients with sick sinus syndrome. Modified from Härtel and Talvensaari (Härtel & Talvensaari, 1975) and Benditt *et al*. (Benditt *et al.*, 1995). **D**, mean (± SEM) density of $I_f$ at -150 mV from sinus node cells isolated from mice of different ages. **E**, representative $I_f$ families normalised to cell capacitance recorded from sinus node cells from mice of different ages. **F**, expression of a range of transcripts in ~24-month-old mice as a percentage of that in ~2-month-old mice as measured by RNAseq (n=8/8). Left, ion channel transcripts involved in pacemaking; right, major histocompatibility complex Class 2 transcripts.

**Fig. 7. Heart failure-dependent nodal dysfunction. A** and **B**, mean (± SEM) normal (A) and intrinsic (B) heart rates in a group of control subjects and patients with non-valvular heart disease (n=17; left) and aortic stenosis (n=17; right) and varying degrees of heart failure (New York Heart Association classes I-IV). Intrinsic heart rate measured after autonomic blockade with propranolol and atropine. Plotted using data from Jose *et al.* (Jose & Taylor, 1969). **C**, arrhythmia incidence over the course of two years in 297 post-myocardial infarction patients fitted with an implantable loop recorder. Plotted using data from Bloch Thomsen *et al.* (Bloch Thomsen *et al.*, 2010). **D**, association

between development of arrhythmias and cardiac and all-cause mortality. Unadjusted hazard ratios calculated using Cox regression analysis. Plotted using data from Bloch Thomsen *et al.* (Bloch Thomsen *et al.*, 2010).

**Fig. 8. Molecular pathways responsible for sinus node dysfunction in heart failure. A**, expression of miR-370-3p (left panel) and *Hcn4* mRNA (centre panel) in the sinus node and the heart rate (measured in conscious animals; right panel) of control mice given vehicle, heart failure mice given vehicle and heart failure mice given antimiR-370-3p. The heart rate is plotted in the days following transverse aortic constriction (TAC) surgery to induce heart failure. **B**, *Hcn4* mRNA expression, *in vivo* intrinsic heart rate, and corrected sinus node recovery time (SNRTc) in control sham-operated rats, heart failure rats, and heart failure rats treated with bisoprolol. Heart failure was induced by an abdominal arterio-venous shunt (volume overload). **C**, hypothesis of events in heart failure. In A and B, means ± SEM plotted. A and C modified from Yanni *et al.* (Yanni *et al.*, 2020) and B modified from Du *et al.* (Du *et al.*, 2016).

**Fig. 9. Downregulation of HCN expression in the sinus node in pulmonary hypertension. A**, mean (± SEM) expression of *Hcn1* and *Hcn4* mRNA in the right ventricle, right atrium and sinus node of control rats (left bars) and monocrotaline-injected rats with pulmonary hypertension (right bars). **B**, thin sections through the sinus node from a control rat and a monocrotaline-injected rat with pulmonary hypertension immunolabelled for HCN4. From Yamanushi *et al.* (Yamanushi *et al.*, 2010).

**Fig. 10. Downregulation of HCN expression and $I_f$ in the sinus node in "AF". A**, mean (± SEM) *Hcn2* and *Hcn4* mRNA expression in the right atrial free wall and sinus node of control dogs (left bars) and "AF" dogs (subjected to 7-day atrial tachypacing; right bars). **B**, families of $I_f$ recordings normalised to cell capacitance from sinus node cells isolated from a control dog and an "AF" dog and mean (± SEM) current density-voltage relationships from control and "AF" dogs. Modified from Yeh *et al.* (Yeh *et al.*, 2009).

**Fig. 11. Circadian rhythm in *Hcn4* in the AV node and downregulation of *Hcn4* in the AV node in pulmonary hypertension. A**, mean (± SEM) expression of *Hcn4* mRNA in the mouse sinus node at four time points over 24 h. The final point is a repeat of the point at ZT 0. The data are fitted with a sine wave. **B**, mean (± SEM) expression of *Hcn4* mRNA at the AV junction of control rats (black bars) and monocrotaline-injected rats with pulmonary hypertension (red bars). Expression is shown in atrial muscle, tendon of Todaro, inferior nodal extension, compact node, penetrating bundle and ventricular muscle.

**Fig. 12. Summary. A**, changes in HCN4 expression in health and disease. The size of the HCN4 text is a qualitative indicator of the expression level. From Boyett and D'Souza (Boyett & D'Souza, 2020). **B**, summary of known and putative factors controlling HCN4 transcription and translation covered in this review.

Fig. 1

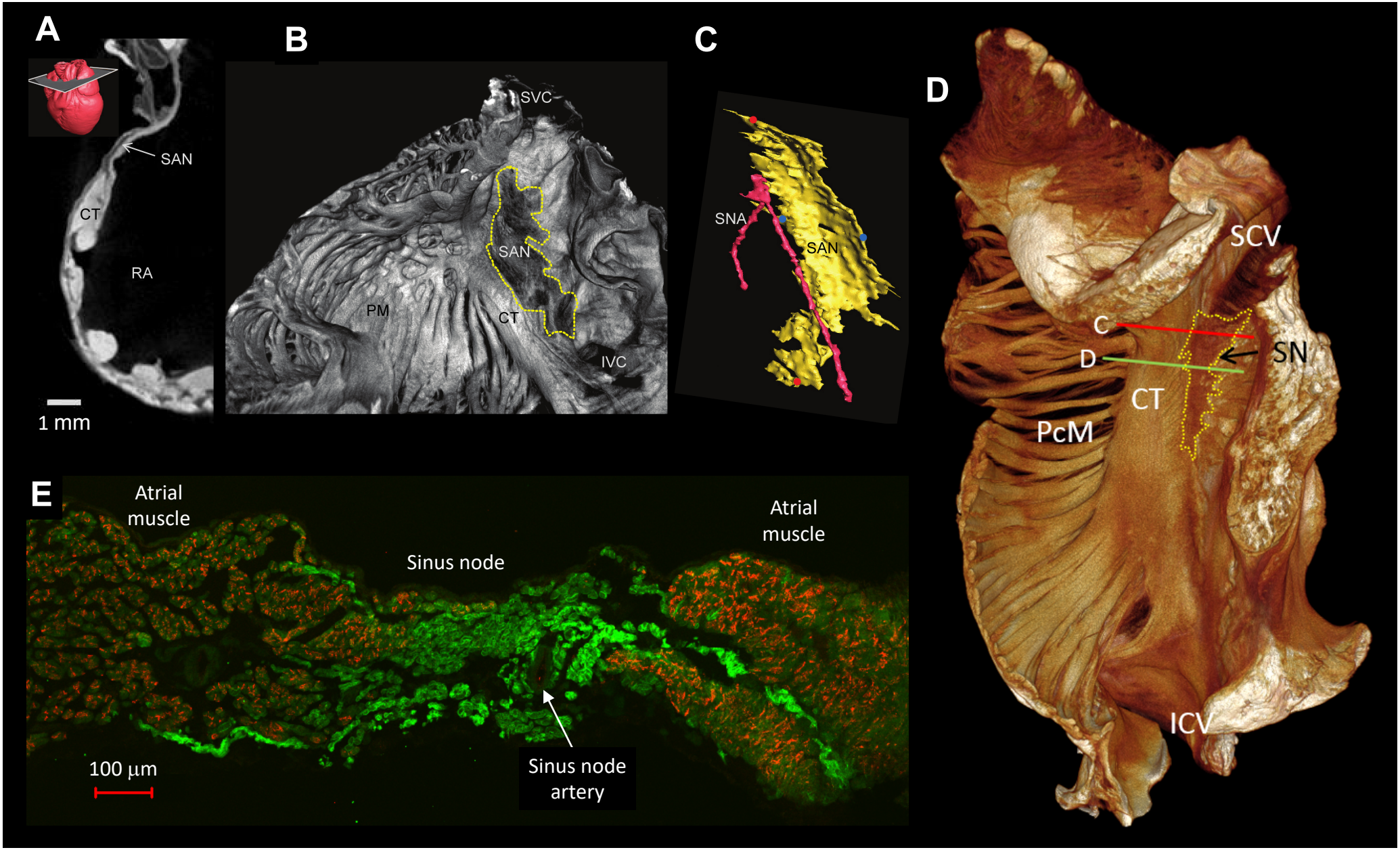
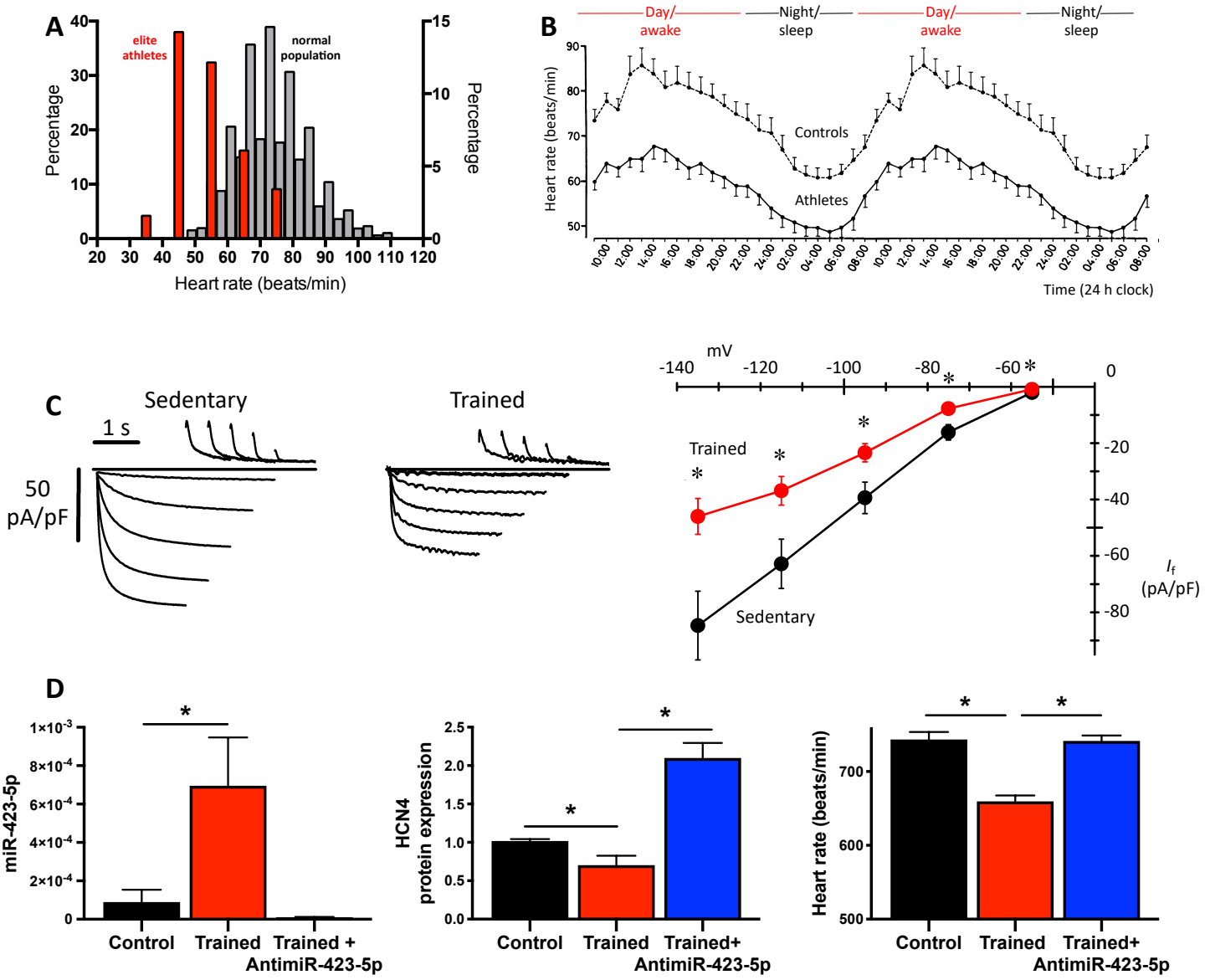
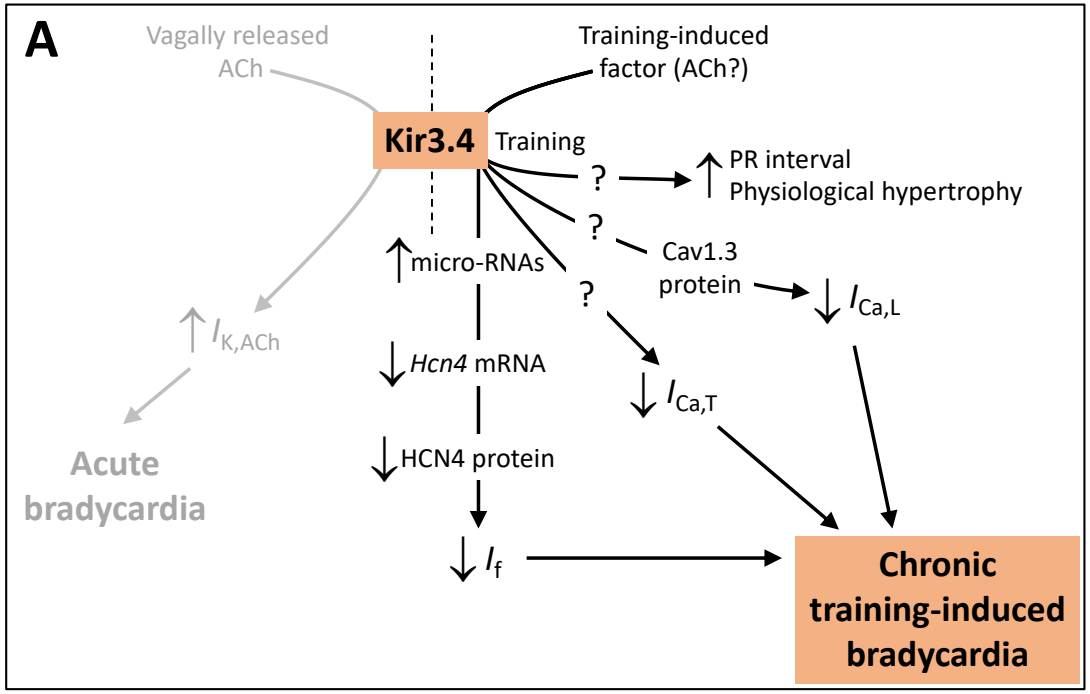

A
SAN
CT
RA
1 mm
B
SVC
SAN
PM
CT
IVC
C
SNA
SAN
D
SCV
C
D
SN
CT
PcM
ICV
E
Atrial muscle
Sinus node
Atrial muscle
100 µm
Sinus node artery

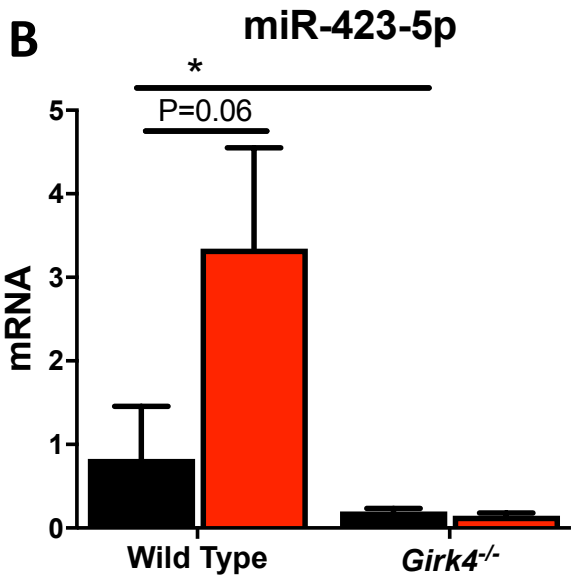
**Fig. 2**

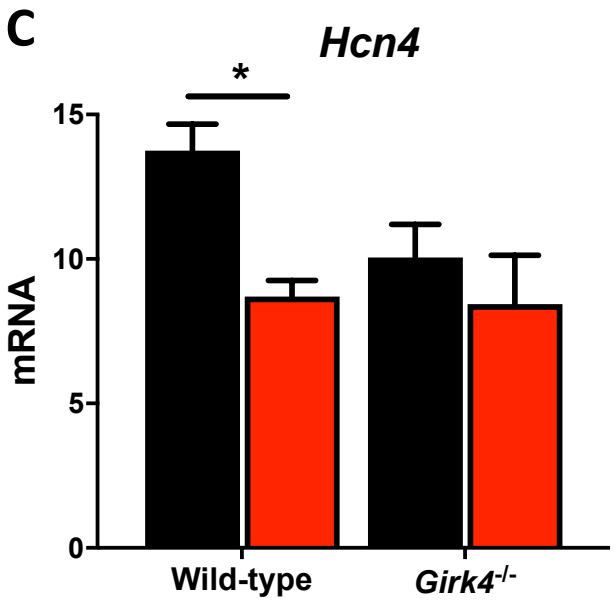

A
elite athletes
normal population
Percentage
Percentage
Heart rate (beats/min)
B
Day/ awake
Night/ sleep
Day/ awake
Night/ sleep
Heart rate (beats/min)
Controls
Athletes
Time (24 h clock)
C
Sedentary
Trained
1 s
50 pA/pF
mV
Trained
Sedentary
$I_f$ (pA/pF)
D
miR-423-5p
Control
Trained
Trained + AntimiR-423-5p
HCN4 protein expression
Control
Trained
Trained+ AntimiR-423-5p
Heart rate (beats/min)
Control
Trained
Trained+ AntimiR-423-5p

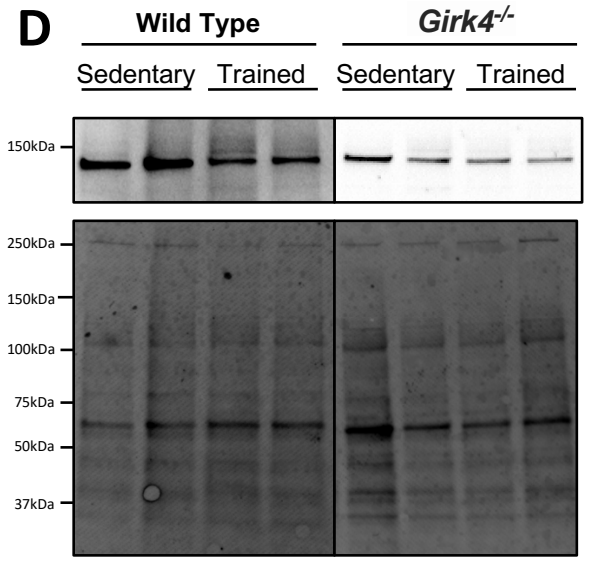
Sedentary (control) Exercise-trained

Fig. 3



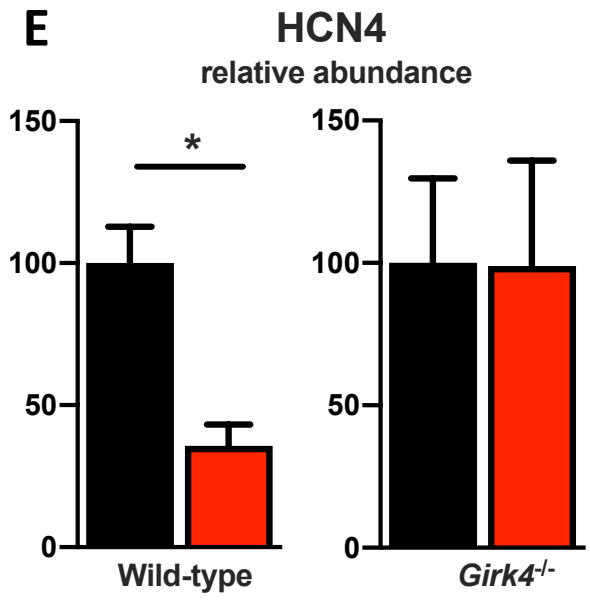



Fig. 4

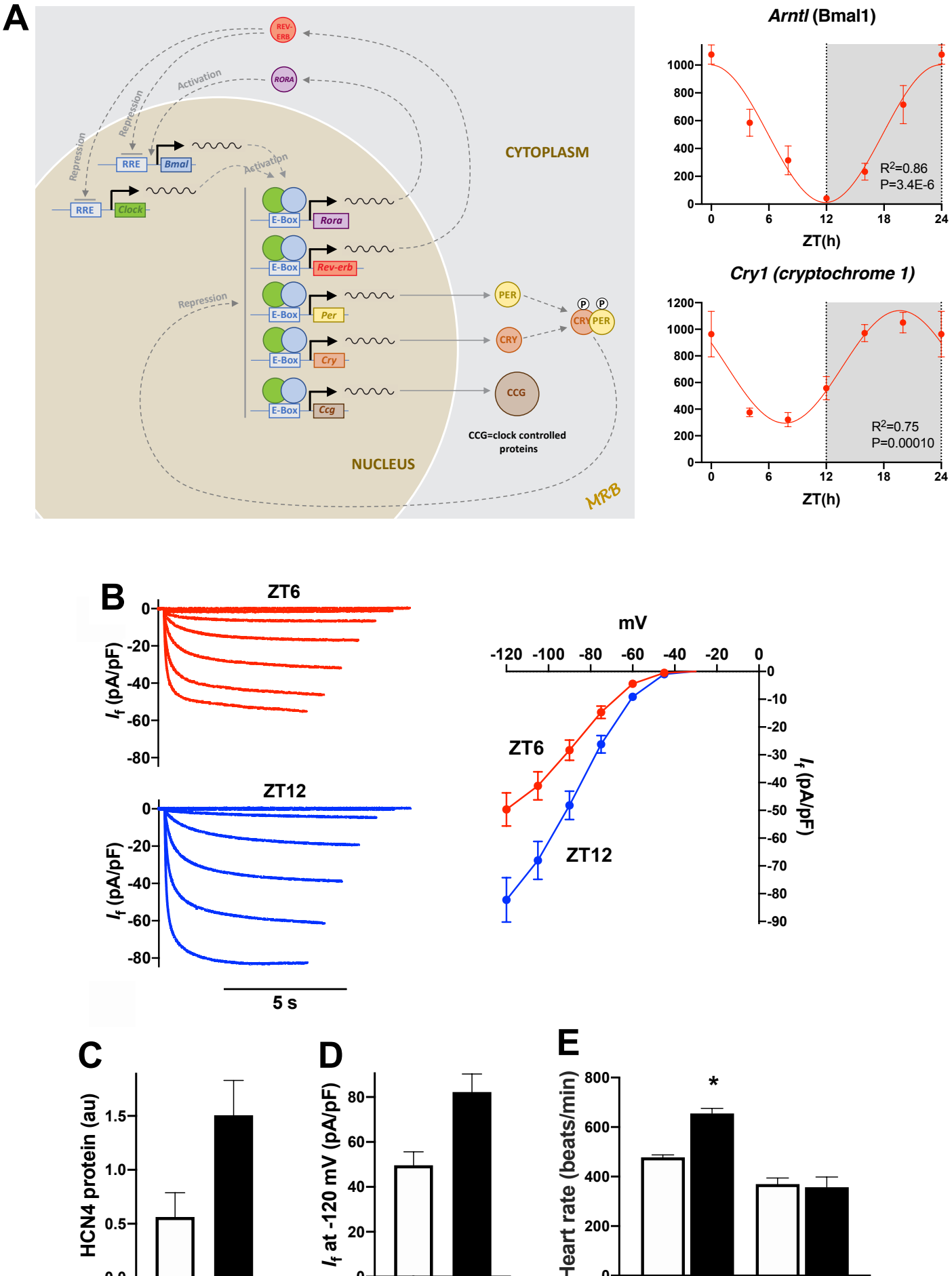

A
REV-ERB
RORA
Activation
Repression
RRE
Bmal
Clock
E-Box
Rora
Rev-erb
Per
Cry
Ccg
PER
CRY
CCG
CCG=clock controlled proteins
CYTOPLASM
NUCLEUS
MRB
Arntl (Bmal1)
R²=0.86
P=3.4E-6
ZT(h)
Cry1 (cryptochrome 1)
R²=0.75
P=0.00010
ZT(h)
B
ZT6
ZT12
I_f (pA/pF)
5 s
mV
C
HCN4 protein (au)
ZT6
ZT12
D
I_f at -120 mV (pA/pF)
ZT6
ZT12
E
Heart rate (beats/min)
Baseline
Ivabradine

Fig. 5

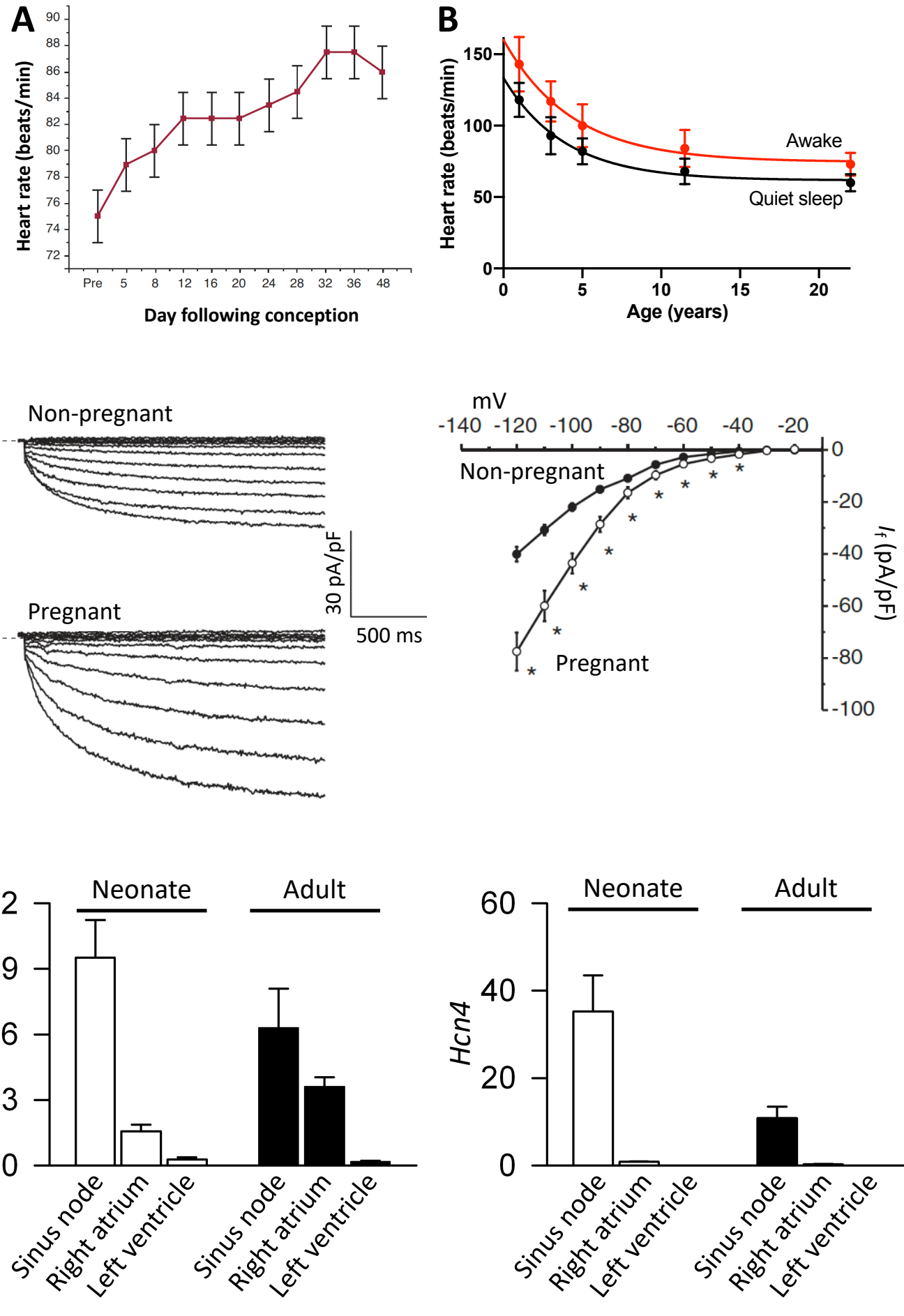

A
Heart rate (beats/min)
90
88
86
84
82
80
78
76
74
72
Pre
5
8
12
16
20
24
28
32
36
48
Day following conception
B
Heart rate (beats/min)
150
100
50
0
Awake
Quiet sleep
0
5
10
15
20
Age (years)
C
Non-pregnant
Pregnant
30 pA/pF
500 ms
mV
-140
-120
-100
-80
-60
-40
-20
Non-pregnant
Pregnant
0
-20
-40
-60
-80
-100
$I_f$ (pA/pF)
D
Hcn1
12
9
6
3
0
Neonate
Adult
Sinus node
Right atrium
Left ventricle
Hcn4
60
40
20
0
Neonate
Adult
Sinus node
Right atrium
Left ventricle

Fig. 6

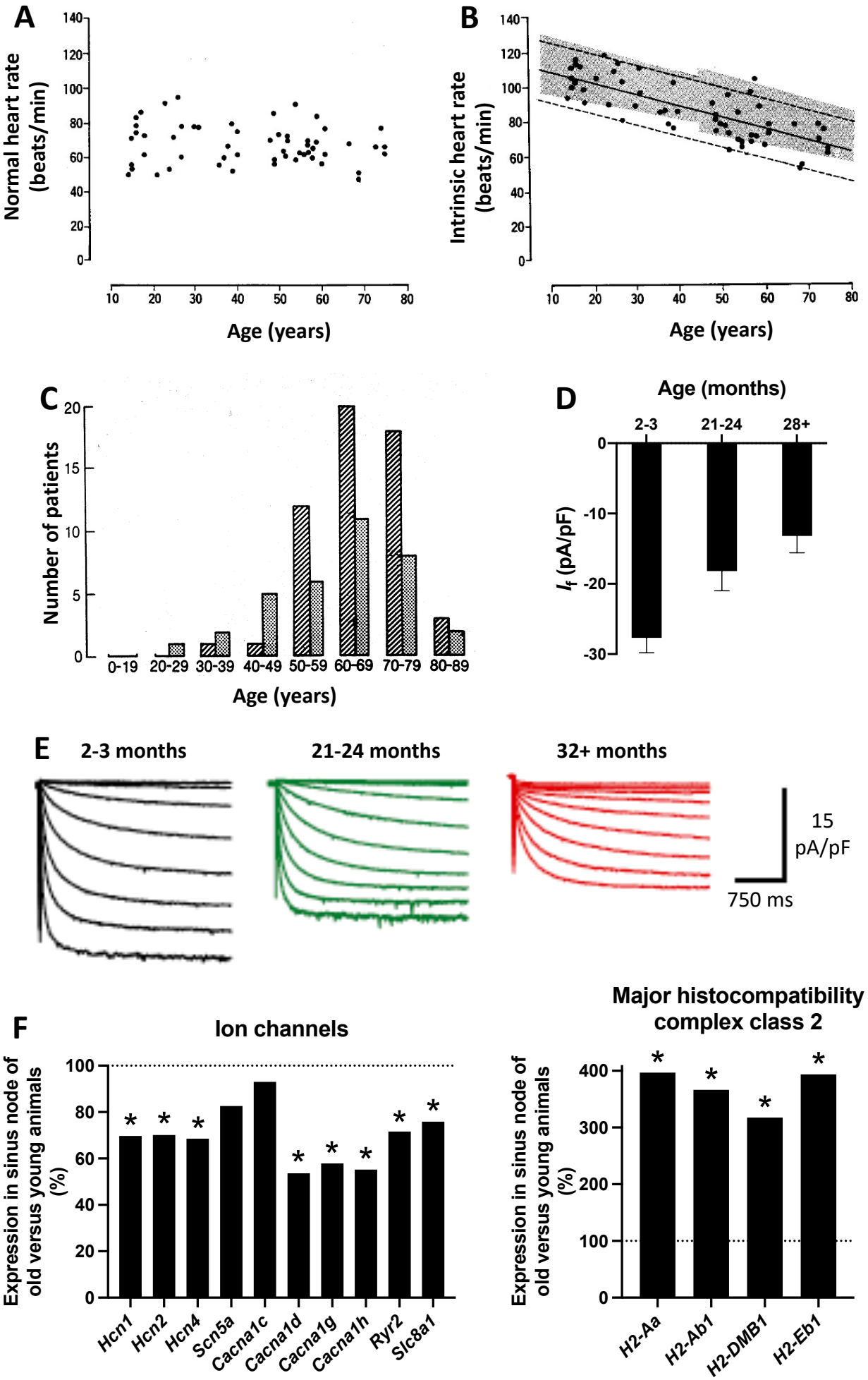

A
Normal heart rate (beats/min)
Age (years)
B
Intrinsic heart rate (beats/min)
Age (years)
C
Number of patients
0-19
20-29
30-39
40-49
50-59
60-69
70-79
80-89
Age (years)
D
Age (months)
2-3
21-24
28+
$I_f$ (pA/pF)
E
2-3 months
21-24 months
32+ months
15 pA/pF
750 ms
F
Ion channels
Expression in sinus node of old versus young animals (%)
Hcn1
Hcn2
Hcn4
Scn5a
Cacna1c
Cacna1d
Cacna1g
Cacna1h
Ryr2
Slc8a1
Major histocompatibility complex class 2
Expression in sinus node of old versus young animals (%)
H2-Aa
H2-Ab1
H2-DMB1
H2-Eb1

Fig. 7

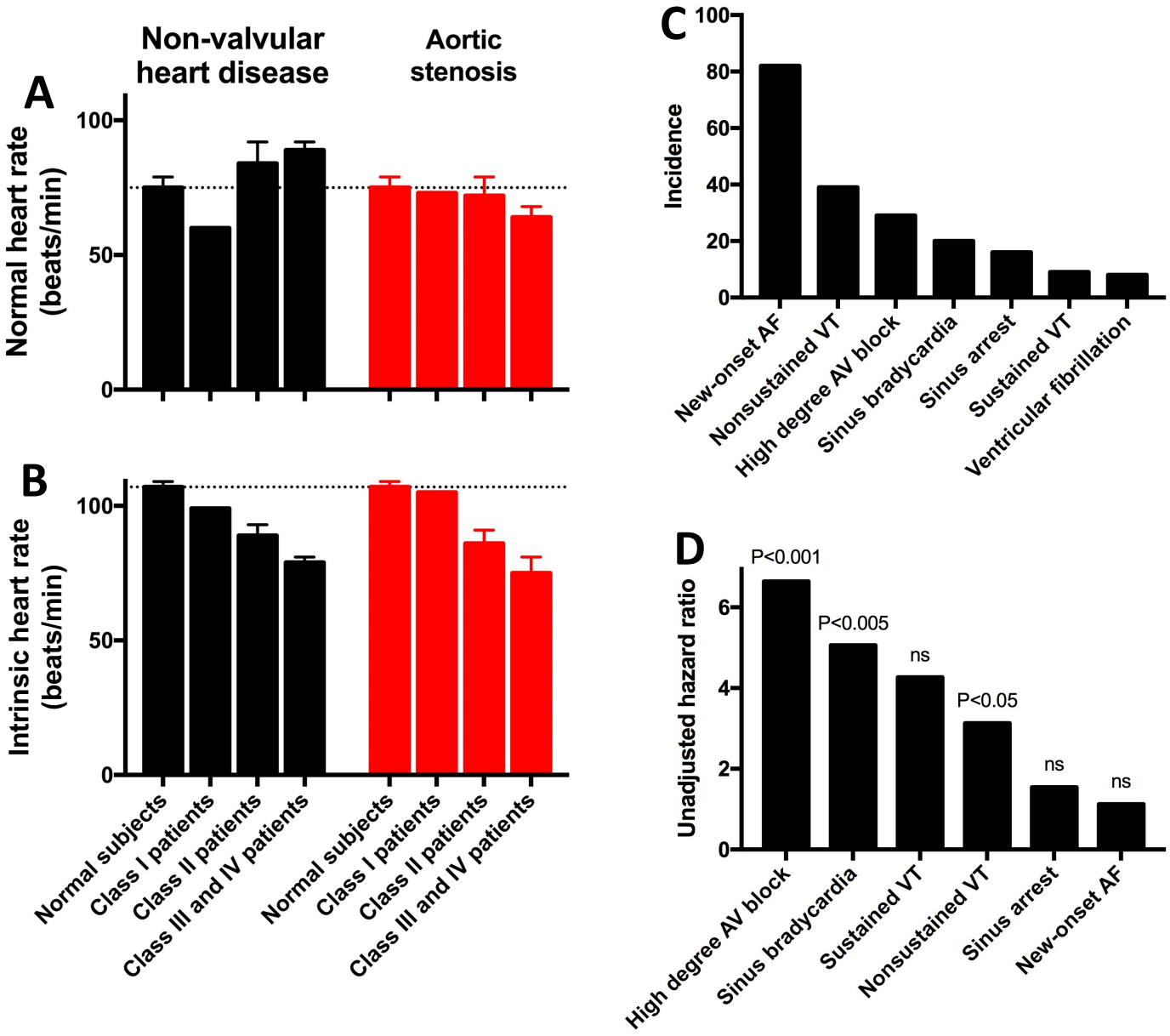

A
Non-valvular heart disease
Aortic stenosis
Normal heart rate (beats/min)
100
50
0
B
Intrinsic heart rate (beats/min)
100
50
0
Normal subjects
Class I patients
Class II patients
Class III and IV patients
Normal subjects
Class I patients
Class II patients
Class III and IV patients
C
Incidence
100
80
60
40
20
0
New-onset AF
Nonsustained VT
High degree AV block
Sinus bradycardia
Sinus arrest
Sustained VT
Ventricular fibrillation
D
Unadjusted hazard ratio
6
4
2
0
P<0.001
P<0.005
ns
P<0.05
ns
ns
High degree AV block
Sinus bradycardia
Sustained VT
Nonsustained VT
Sinus arrest
New-onset AF

Fig. 8

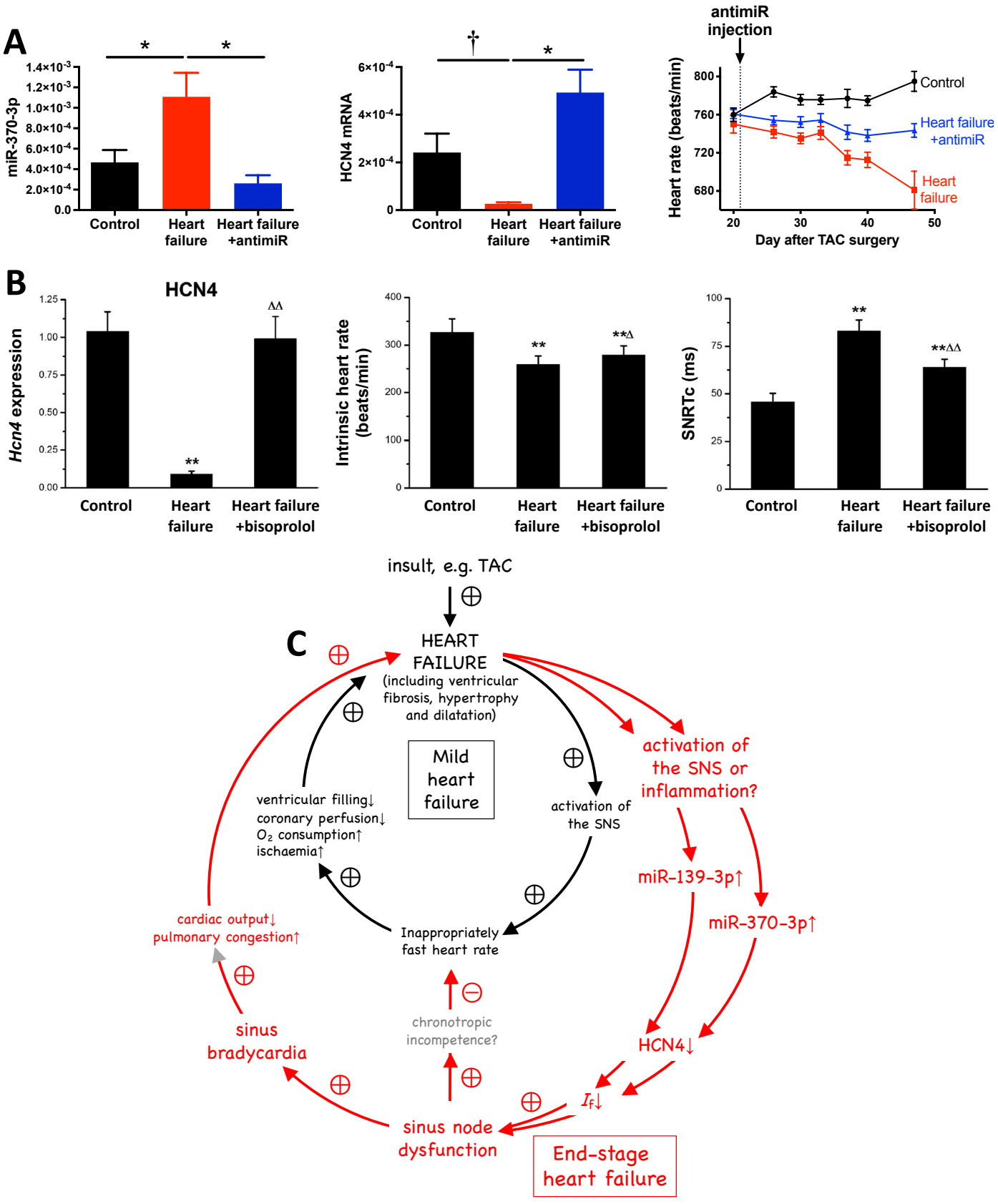

A
miR-370-3p
1.4×10-3
1.2×10-3
1.0×10-3
8.0×10-4
6.0×10-4
4.0×10-4
2.0×10-4
0.0
Control
Heart failure
Heart failure +antimiR
HCN4 mRNA
6×10-4
4×10-4
2×10-4
0
antimiR injection
Heart rate (beats/min)
800
760
720
680
20
30
40
50
Day after TAC surgery
Control
Heart failure +antimiR
Heart failure
B
HCN4
Hcn4 expression
1.25
1.00
0.75
0.50
0.25
0.00
Intrinsic heart rate (beats/min)
400
300
200
100
0
SNRTc (ms)
100
75
50
25
0
Heart failure +bisoprolol
C
insult, e.g. TAC
HEART FAILURE
(including ventricular fibrosis, hypertrophy and dilatation)
Mild heart failure
activation of the SNS or inflammation?
activation of the SNS
ventricular filling↓
coronary perfusion↓
O2 consumption↑
ischaemia↑
miR-139-3p↑
miR-370-3p↑
cardiac output↓
pulmonary congestion↑
Inappropriately fast heart rate
chronotropic incompetence?
HCN4↓
If↓
sinus bradycardia
sinus node dysfunction
End-stage heart failure

Fig. 9

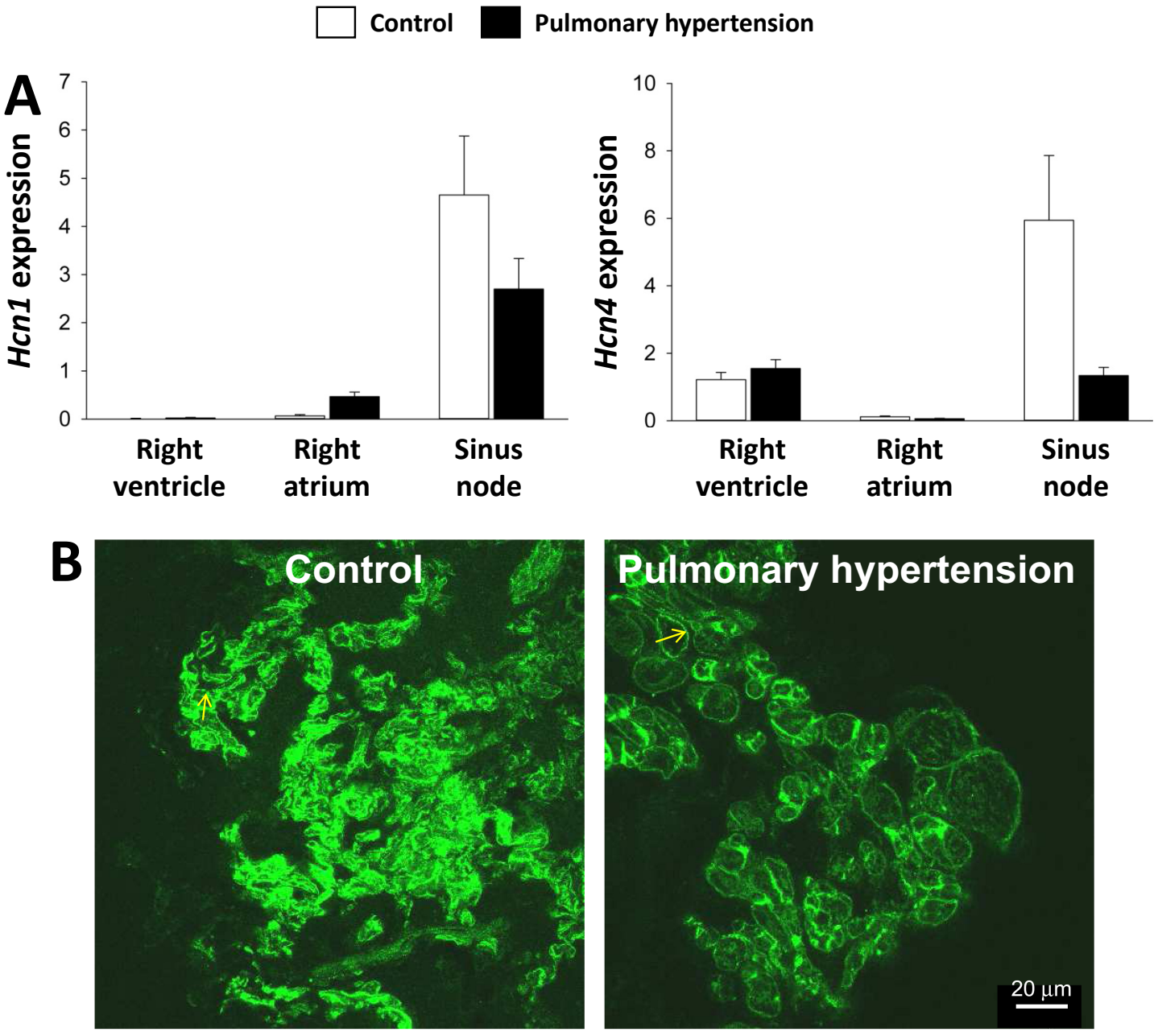
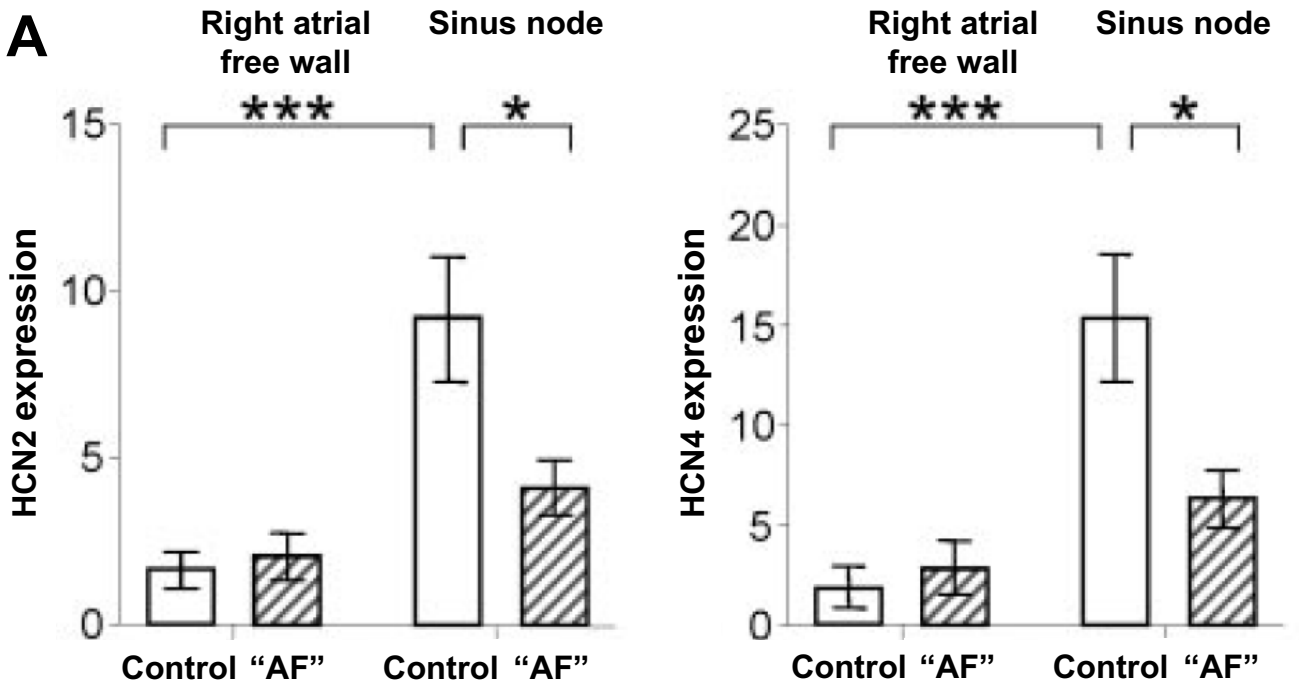

Control
Pulmonary hypertension
A
Hcn1 expression
0
1
2
3
4
5
6
7
Right ventricle
Right atrium
Sinus node
Hcn4 expression
0
2
4
6
8
10
Right ventricle
Right atrium
Sinus node
B
Control
Pulmonary hypertension
20 µm

Fig. 10


A
Right atrial free wall
Sinus node
***
*
HCN2 expression
15
10
5
0
Control
"AF"
Control
"AF"
Right atrial free wall
Sinus node
***
*
HCN4 expression
25
20
15
10
5
0
Control
"AF"
Control
"AF"

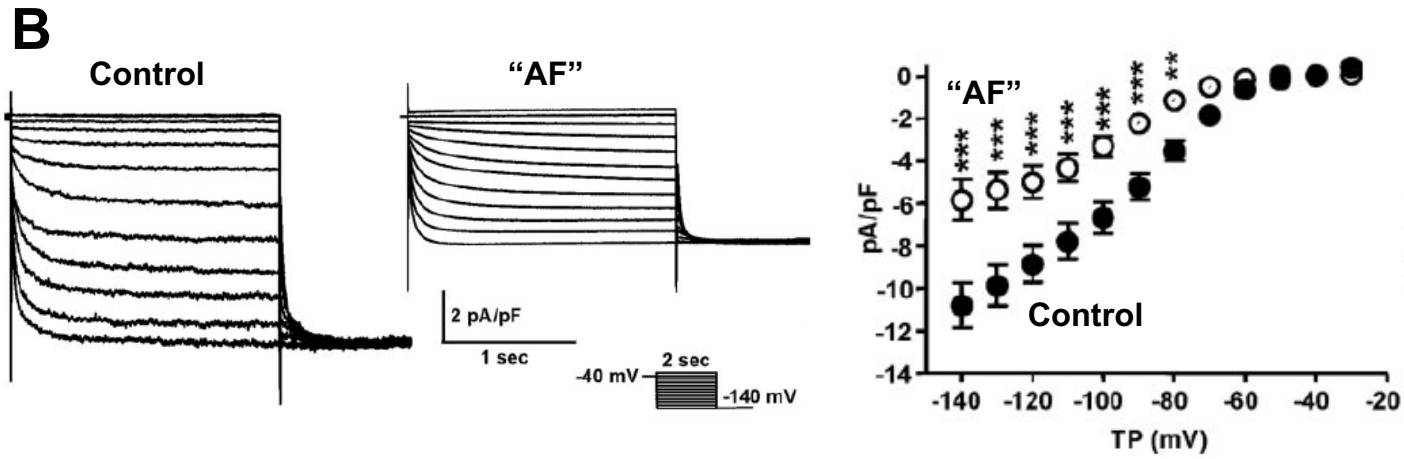

B
Control
"AF"
2 pA/pF
1 sec
2 sec
-40 mV
-140 mV
"AF"
Control
pA/pF
0
-2
-4
-6
-8
-10
-12
-14
-140
-120
-100
-80
-60
-40
-20
TP (mV)

Fig. 11

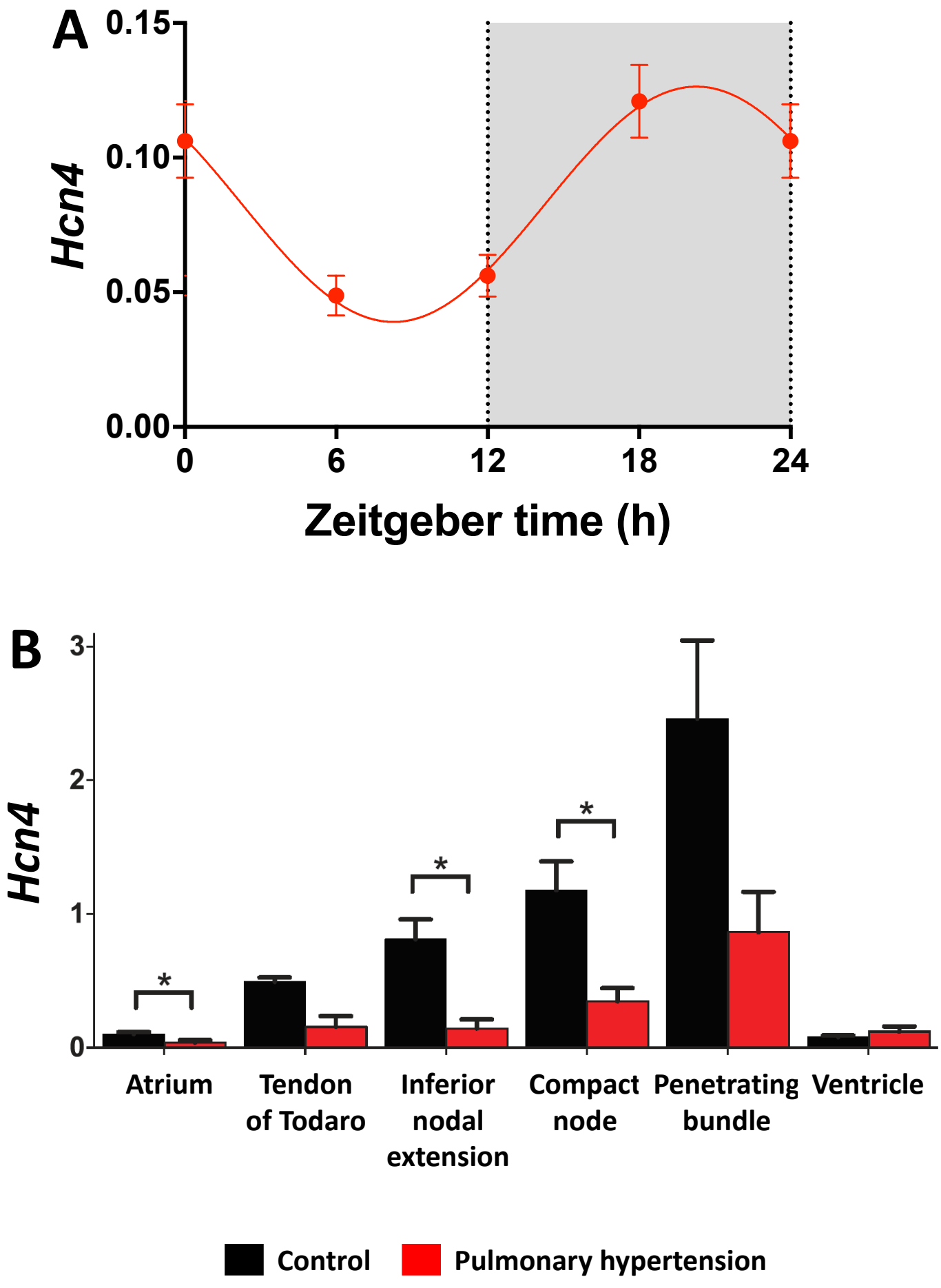
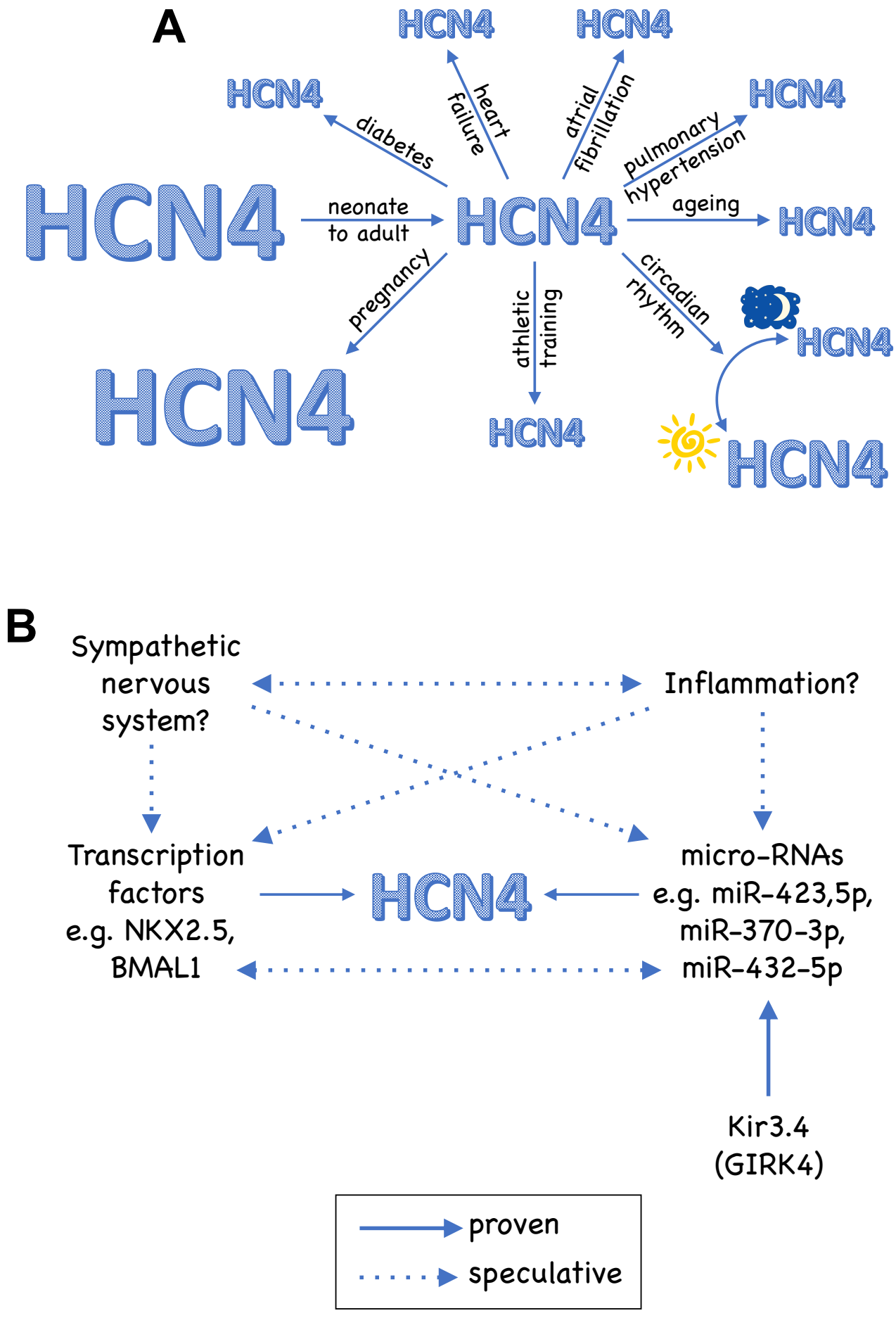

A
Hcn4
0.15
0.10
0.05
0.00
0
6
12
18
24
Zeitgeber time (h)
B
Hcn4
3
2
1
0
*
*
*
Atrium
Tendon of Todaro
Inferior nodal extension
Compact node
Penetrating bundle
Ventricle
Control
Pulmonary hypertension

Fig. 12


A
HCN4
HCN4
HCN4
HCN4
HCN4
HCN4
HCN4
HCN4
HCN4
HCN4
HCN4
HCN4
diabetes
heart failure
atrial fibrillation
pulmonary hypertension
neonate to adult
ageing
pregnancy
athletic training
circadian rhythm
B
Sympathetic nervous system?
Inflammation?
Transcription factors e.g. NKX2.5, BMAL1
HCN4
micro-RNAs e.g. miR-423,5p, miR-370-3p, miR-432-5p
Kir3.4 (GIRK4)
proven
speculative