**Full title:** Why do athletes have AV block? A new role for electrical remodelling of the atrioventricular node in equine and murine models of endurance exercise

**Running title:** Mesirca; Electrical remodelling of the AV node in athletes

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**ABSTRACT**

**Rationale:** Athletes present with atrioventricular node (AV node) dysfunction manifesting as AV block. This can necessitate electronic pacemaker implantation, known to be more frequent in athletes with a long training history.

**Objective:** AV block in athletes is attributed to high vagal tone. Here we investigated the alternative hypothesis that electrical remodelling of the AV node is responsible.

**Methods and results**: Radio-telemetry ECG data and AV node biopsies were collected in sedentary and trained Standardbred racehorses, a large-animal model of the athlete’s heart. Trained horses presented with longer PR intervals (that persisted under complete autonomic block) *versus* sedentary horses, concomitant with reduced expression of key ion channels involved in AV node conduction: L-type Ca2+ channel subunit CaV1.2 and the hyperpolarization-activated cyclic nucleotide gated channel 4 (HCN4). AV node electrophysiology was explored further in mice; prolongation of the PR interval (*in vivo* and *ex vivo*), Wenckebach cycle length and AV node refractory period was observed in mice trained by swimming *versus* sedentary mice. Transcriptional profiling in laser-capture microdissected AV node revealed striking reduction in pacemaking ion channels in trained mice, translating into protein downregulation of CaV1.2 and HCN4. Correspondingly, patch clamp recordings in isolated AV node myocytes demonstrated a training-induced reduction in *I*Ca,*L* and *I*f density that likely contributed to the observed lower frequency of action potential firing in trained cohorts. microRNA (miR) profiling and *in vitro* studies revealed miR-211-5p and miR-432 as direct regulators of CaV1.2 and HCN4. *In vivo* miRs suppression or detraining restored training-induced PR prolongation and ion channel remodelling.

**Conclusions:** Training-induced AV node dysfunction is underscored by likely miR-mediated transcriptional remodelling that translates into reduced current density of key ionic currents involved in impulse generation and conduction. We conclude that electrical remodelling is a key mechanism underlying AV block in athletes.

**Keywords:** AV block, Athletic training, Electrophysiology, Ion channels, microRNAs

**Non-standard abbreviations:** AV, atrioventricular node; AVERP, atrioventricular node effective refractory period; τ, time constant; miR, microRNA

**INTRODUCTION**

Despite its many well-recognised cardiovascular benefits, it is now accepted that sustained endurance exercise leads to the development of cardiac arrhythmias including atrial fibrillation,1-5 sinus bradycardia6-8 and atrioventricular (AV) block (also known as heart block).6, 9, 10 AV block (impairment of conduction through the AV node), is a common occurrence in athletes, manifesting as a longer PR interval (first degree AV block), 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).10-12 AV block in athletes has been correlated to the intensity and length of the training period;13 in veteran endurance athletes, the prevalence of first- and second-degree AV block has been recorded as ranging from 28–45% and 15–40% respectively,14 in contrast to 0.60% and 0.03% in the general population.15 Several studies have also noted the presence of asystolic pauses in athletes that last from 1.6 to 15 s.7, 8, 16 In athletes, first-degree and type-I second-degree AV block are generally considered as benign physiological changes; however, recent large-scale clinical studies have concluded that type-I second-degree AV block increases the risk of atrial fibrillation and pacemaker implantation in later life.17-19 AV block may also present symptomatically; in second-degree AV block, conduction failure through the AV node can result in syncope or palpitations.20 These observations likely contribute to the increased incidence of electronic pacemaker implantation reported in veteran athletes.6, 21

Despite this evidence of a burden of AV node dysfunction in veteran athletes, there is very limited study of the underlying mechanisms. AV block in athletes is currently attributed to the autonomic nervous system–a supposed increase in vagal tone induced by training,22 even though vagal tone has never been directly measured in athletes.23 Furthermore, previous work in human athletes has demonstrated that AV node dysfunction in trained athletes *versus* sedentary subjects (typified by a prolonged Wenckebach cycle length and AV node effective refractory period, AVERP),persisted even under complete pharmacological block of the autonomic nervous system.10 This implicates a role for intrinsic training-induced electrophysiological alterations, as we have previously described in the sinus node of human athletes24 and rodent models.24, 25 Here, using equine and rodent models of endurance training combined with *in vivo*, *in vitro* and *in silico* approaches, we demonstrate for the first time, a predominant role for intrinsic electrical remodelling in training-induced AV node dysfunction. We go on to show that AV node ion channel expression is under the control of the miR network and test the therapeutic potential of targeting noncoding miRs to rescue AV node dysfunction

**METHODS**

Detailed methods are given in the Online Data Supplement. 24-hour Holter recordings were obtained from sedentary, trained and detrained Standardbred trotters. PR intervals were measured in horses at baseline and after complete block of the autonomic nervous system by intravenous injection of 0.04 mg/kg atropine and 0.20 mg/kg propranolol. Right atrial biopsies encompassing the AV node were dissected and the component structures of the equine AV conduction axis identified histologically and by immunolabelling. CaV1.2 and HCN4 expression were quantified by immunohistochemistry and western blotting. miRs were assessed by qPCR. Training-induced AV node dysfunction was explored further in mice; in the first set of experiments 8 to 10-week-old male C57BL6/j mice were trained by swimming for 20 weeks to model long-term exercise training in humans. At the end of the training period, in the anaesthetised mouse echocardiography was performed and the electrocardiogram (ECG) was recorded *in vivo* (at baseline and on complete autonomic block achieved by intraperitoneal injection of 0.5 mg/kg atropine and 1 mg/kg propranolol). AV node function was tested *in vivo* (by programmed electrical stimulation) and *ex vivo* (in the isolated, denervated AV node). Right atrial biopsies encompassing the AV node were frozen, cryosectioned and subjected to histology and immunolabelling to enable laser-capture microdissection of AV node and right atrial regions and subsequent qPCR analysis of 96 key mediators of AV node automaticity and conduction. Protein levels of selected ion channels were assessed by immunolabelling, and current density of corresponding ionic currents tested by patch clamp in isolated AV node myocytes. Patch clamp data were incorporated into a biophysically detailed, one-dimensional model of the AV node to simulate the effect of training on AV node conduction. The effect of the funny current (*I*f) selective blocker ivabradine on the PR interval in trained and sedentary animals was tested *in vivo*. The role of miRs in training-induced AV node transcriptional remodelling was assessed by profiling the expression levels of 750 miRs. Specific interactions between dysregulated miRs and selected ion channels was verified by luciferase reporter gene assays and site directed mutagenesis. The effect of overexpression of miR-211-5p and miR-432 on endogenous ion channel transcription was tested by qPCR and western blot in embryonic stem (ES) cell-derived myocytes that expressed Cav1.2 and HCN4.

In the course of the study it was determined that swim-training mice for 60 min, twice per day for four weeks produced analogous prolongation of the PR interval and remodelling of AV node ion channels as observed with the longer training duration described above. In line with the ethical principles of the 3Rs (Replacement, Reduction, Refinement), further studies were carried out in mice trained for this duration, including assessment of (i) training-induced change to *I*K and action potential morphology and duration in isolated myocytes with patch clamp and (ii) the impact of *in vivo* suppression of miR-211-5p and miR-432 on training-induced PR prolongation. The latter was achieved by administering a combination of cholesterol-conjugated antimiRs followed by *in vivo* electrophysiology, Western blot and qPCR to characterise the mice and study CaV1.2 and HCN4 remodeling. Finally, the effect of detraining on the ECG and transcript expression of CaV1.2 and HCN4 was assessed following a detraining period of 12 weeks. Statistically significant differences were determined using an appropriate test- See Statistical Analysis Table; P<0.05 was regarded as significant.

**RESULTS**

**Evidence of AV node dysfunction and ion channel remodelling in trained racehorses** Experiments were conducted on trained (n=13) and sedentary (n=6) horses, a high fidelity model of the athlete’s heart (further explored in the **Discussion**).26, 27 First, ECGs from trained and sedentary horses were analysed to establish training-induced changes. **Figure 1A** illustrates a typical ECG recording from a sedentary horse. The ECG has a QRS complex following every P wave; in **Figure 1B-D** representative traces from trained racehorses are given, illustrating variations of second-degree AV block, characterised by intermittent conduction block and absent QRS complexes. Note that in this work we did not distinguish between type I and type II second-degree AV block (considered further in the Methods section of the Data Supplement). **Figure 1E** shows the resting heart rate under baseline conditions and, as expected, the heart rate was significantly lower in trained horses. Further analysis revealed that trained racehorses also presented with a significantly longer PR interval **(Figure 1F)** and a trend towards an increase in the number of episodes of second-degree AV block compared to sedentary horses **(Figure 1G).** The effect of detraining on the ECG changes was investigated by comparing trained horses with a separate cohort of horses (n=5) that had been detrained for varying durations between 2 - 60 months. The ECG changes were reversible on detraining: the resting heart rate increased to that typical of sedentary horses in an exponential manner with a time constant, τ, of 10.3 months (**Figure 1H**; R2=0.69); the PR interval declined with a τ = 2.5 months (**Figure 1I**; R2=0.31). These data show that in horses, as in humans, exercise training induces reversible sinus bradycardia and PR prolongation.

24 h Holter recordings were obtained from a further cohort of 5 trained and 5 sedentary horses to study the natural variation of heart rate and the PR interval under resting conditions. Trained horses presented with slower minimal and maximal heart rates (given in beats/min {bpm}; minimum, 30±2 bpm; maximum, 43±2 bpm) compared to sedentary horses (minimum, 32±1 bpm; maximum, 49±2 bpm). Notably, as given in **Figure 1J**, prolongation of the PR interval in trained horses was apparent at both minimum (325.4±11.1 ms *versus* 380.6±27.3 ms) and maximum (297.8±7.1 ms *versus* 326.2±11.8 ms) heart rates. A mixed effects linear model showed that the PR interval was longer both at slower heart rates (P=2.1 x 10-3) and in trained animals (P=8.21 x 10-2); however, the interaction between heart rate and training status was not significant (P=1.82 x 10-1). This analysis indicates that the difference in the PR interval between sedentary and trained horses in this cohort does not depend upon the underlying heart rate. Importantly, previous characterisation of the intrinsic rate-dependent properties of the AV node have unequivocally demonstrated that a faster basic rate produces an intrinsically longer AV node conduction time.28-30 Therefore, in this study the intrinsic training-induced slowing of PR conduction cannot be explained by the underlying heart rate difference between sedentary and trained animals.

The training-induced AV node dysfunction could be the result of altered autonomic nerve activity to the heart (for example, increased vagal tone) or a change in the intrinsic properties of the AV node. To test the involvement of the autonomic nervous system, sedentary and trained were subjected to complete autonomic blockade. Autonomic blockade was achieved by intravenous injection of 0.20 mg/kg propranolol and 0.04 mg/kg atropine. Doses of atropine and propranolol were based on Hamlin *et al*.,31 who made dose-response measurements in healthy horses and showed complete block for up to 45 min under agonist challenge. **Figure 1K shows** that the prolongation of the PR interval induced by training persisted under complete block of the autonomic nervous system (262.8±6.5 ms in sedentary horses *versus* 274.0±2.0 ms in trained horses) whereas no incidences of high-degree AV block were recorded under conditions of autonomic blockade. We thus conclude that in this study first degree AV block in trained horses under resting conditions cannot be fully attributed to autonomic influences and intrinsic mechanisms must be considered.

Potential intrinsic mechanisms underlying AV node dysfunction were investigated in AV node tissue from sedentary and trained horses. The precise location and structure of the AV node in horses has not been established and Masson’s trichrome staining was used to identify key histological features of the horse AV conduction axis. **Figure 1L** shows the compact node, morphologically defined by its position adjacent to the musculature of the AV septum. The small, packed cells of the compact node were interwoven within a matrix of connective tissue and bordered by transitional cells that distinguished it from the longitudinally oriented working atrial myocardium. The serial sectionscontinued inferiorly through the conduction axis illustrating the penetrating bundle of His, the left and right bundle branches and Purkinje fibres that were embedded deep within connective tissue (data not shown). As increased extracellular matrix deposition is commonly invoked as a mechanism underlying slowed AV node conduction,32 collagen deposition in the AV node was assessed in immunolabelled sections using ZEN Intellesis (Zeiss) - a machine learning-based tool that enabled image segmentation and pixel classification to identify the extracellular matrix. Representative images given in **Online Figure IA-B** demonstrate that the collagen area fraction was not altered in sedentary *versus* trained horses.

Next, the role of ion channel remodelling within the AV node was investigated. Histological identification was further supplemented by immunohistochemical labelling with antibodies against HCN4 to confirm the presence of AV node myocytes (**Figure 1M,** top panels). HCN channels carry *I*f, an inward current flowing during diastole that constitutes an important mechanism for phase 4 depolarization and pacemaking.33 The role of *I*f in conduction through the AV node is underappreciated, although several studies suggest that it is involved34-39 (explored further in the **Discussion**). Intriguingly, image analysis of HCN4 immunolabelled images revealed a reduction in HCN4 protein expression in the compact node of trained (n=7) *versus* sedentary (n=7) horses (**Figure 1M, top panels, and Figure 1N**). The reduction of HCN4 in the trained compact node was confirmed by western blot in biopsies of the compact node from sedentary (n=5) and trained (n=5) horses. A representative blot is shown in **Figure 1O** (top panel). HCN4 protein expression (top band) was normalised to total protein expression (**Figure 1O, lower panel**) and is plotted in **Figure 1P** – there was a reduction in normalised HCN4 band intensity in AV node biopsies from trained *versus* sedentary horses.

The inward current carrying L-type Ca2+ channels are known to play a major role in AV node diastolic depolarization, setting excitability and the upstroke of the action potential. Cav1.2 and Cav1.3 are important Ca2+ channels in the heart and their knockout has been reported to slow or block AV node conduction.36, 38, 40 Here we investigated protein expression of the key L-type Ca2+ channel subunit Cav1.2 (commercially sourced antibodies against Cav1.3 lacked specificity and could not be validated in horse tissue). Akin to HCN4, we observed a significant reduction in signal intensity of Cav1.2 immunolabelling in trained (n=7) *versus* sedentary (n=7) horses(**Figure 1M, bottom panels, Figure 1Q**). Qualitatively similar data were obtained by western blot; the normalised band intensity for CaV1.2 was reduced in compact node biopsies from trained horses (n=5) compared to sedentary (n=5) horses (**Figure 1R,S**).

Taken together, these data are the first demonstration of *intrinsic* electrical remodelling of the AV node in a large animal model of the athlete’s heart and this directly contravenes the commonly accepted mechanisms of high vagal tone or idiopathic fibrosis as primary drivers of training-induced AV node dysfunction.

**Swim training in mice induces AV node dysfunction with widespread transcriptional remodelling of ion channels.**

Training-induced dysfunction of the AV node was explored further in mice. Mice were swim trained for 20 weeks to recapitulate approximately 10 years of exercise in humans.41, 42 As expected, at the end of the training period, trained mice (n=15) presented with sinus bradycardia, mild left ventricle hypertrophy evidenced by echocardiography (**Online** **Table S1**) and a significant prolongation of the PR interval (**Figure 2A**) compared to sedentary mice (n=15). Involvement of the autonomic nervous system in mediating training-induced PR interval prolongation was tested by blocking cardiac muscarinic and β receptors (using 1 mg/kg atropine and 1 mg/kg propranolol - justification of doses given by D’Souza *et al.*43); as observed in horses (**Figure 1K)**,the PR prolongation in trained animals persisted after complete block of the autonomic nervous system (**Figure 2A**) once again suggesting intrinsic training-induced remodelling of the AV node.

To determine how prolonged exercise training impacts AV node conduction, we performed an *in vivo* electrophysiological study to specifically determine: (i) the pacing cycle length at which Wenckebach conduction occurred and (ii) the AV node effective refractory period. **Figure 2B** shows that the Wenckebach cycle length and the AV node effective refractory period were significantly prolonged in trained mice compared to sedentary mice, further evidence of training-induced impairment of AV node conduction. Recordings obtained during *in vivo* programmed electrical stimulation demonstrating AV Wenckebach block in trained and sedentary mice are shown in **Figure 2C**. There was a progressive PR prolongation prior to AV block, which developed at a longer cycle length in trained mice (106 ms) compared to sedentary mice (74 ms). Additionally, and consistent with our previous findings, we also observed sinus bradycardia and an increased sinus node recovery time in trained mice compared to sedentary mice (**Online Figure II**). To determine whether longer PR intervals in trained mice were related to the underlying reduction in heart rate, the PR interval was measured under constant pacing cycle lengths of 100 and 120 ms. As given in **Figure 2D**, trained animals still presented with a significantly longer PR interval *versus* sedentary animals under these conditions.

The AV node is supported *in vivo* by neurohumoral influences, and, therefore, experiments were conducted on isolated denervated right atrial preparations encompassing the AV node (**Figure 2E**). From extracellular potential recordings and consistent with **Online Figure II** we confirmed a prolonged sinus node cycle length in trained mouse preparations (**Figure 2F**). Importantly, as given in the representative trace in **Figure 2G** (left panel)**,** trained mice (n=7) had a significantly prolonged atrial-His (A-H) interval compared to sedentary mice (n=8) that was also apparent under a fixed pacing cycle length of 150 ms (**Figure 2G**, right panel). There was also a tendency towards prolongation of the AVERP (trained, 123.7 ± 12.5 ms; sedentary, 110.8 ± 8.8 ms; n=6/6), Wenckebach cycle length (trained, 146.5 ± 12.4 ms; sedentary, 132.8 ± 7.2 ms; n=4/4), and the H-V interval at a fixed pacing cycle length of 150 ms (trained, 19.69 ± 2.02 ms; sedentary, 18.75 ± 0.75 ms) in the isolated AV node, but these parameters did not reach statistical significance (data not shown). Cumulatively, these studies support a role for intrinsic training-induced adaptations to the AV node.

To understand the mechanisms underlying training-induced AV block, we assessed transcriptional changes in key ion channels, connexins, intracellular Ca2+-handling proteins, ion transporters and extracellular matrix components and mediators that are known to be involved in AV node impulse generation and conduction. Transcriptional profiling of the mouse AV node presents special challenges because of its small size, and the interdigitation of AV node myocytes with the extracellular matrix and ‘working’ myocytes of the atrial and ventricular septae. To obtain an accurate sample of the AV node, right atrial preparations (representative example given in **Figure 2H**) were first cryosectioned and stained with Masson’s trichrome for histological identification of the AV conduction axis (**Figure 2H**). This was followed by immunofluorescent labelling of relevant regions with an anti-HCN4 antibody (**Figure 2H, lower panel**) that guided laser-capture microdissection of HCN4-positive AV node regions such as the compact node (**Figure 2H, inset**).

AV node regions were then processed for medium throughput qPCR assessment of 96 key AV node transcripts using TaqMan Low Density Array Cards. Six reference transcripts were assessed (*Hprt*, *Pgk1, Tbp, Ipo8, Gapdh and 18s* tested) and *Hprt* and *Pgk1* determined to be the optimal combination for normalisation based on stability scores determined by the algorithm GeNorm44 (**Online Figure III**). Differential expression testing using the non-parametric Limma test followed by Benjamini-Hochberg False Discovery Rate correction set at 5% demonstrated a widespread downregulation of mRNAs for Ca2+ channels, intracellular Ca2+-handling molecules, HCN channels, K+ channels and connexins in the AV node of trained mice (**Figure 2I)**. Na+ channels, components of the Na+–K+ pump and components of the extracellular matrix (**Online Figure IE**) were also reduced by training (**Online Table S2 and S3)**. Remarkably, training-induced remodelling appeared to be restricted to the AV node as none of the transcripts assessed were significantly altered in corresponding right atrial tissue collected by laser capture microdissection from sedentary and trained animals (**Online Figure IV**).

 As an aside, pilot studies of Masson’s trichrome stained AV conduction axis (n=3 mice per group) indicated unchanged collagen deposition between groups (**Online Figure IC-D**). These data combined with the transcript downregulation in the major fibrillar collagens (**Online Figure I**) meant that the possibility of a training-induced increase in extracellular matrix deposition was not pursued further as a contributor to AV node dysfunction in mice.

**Role for *I*Ca,*L*, *I*f and *I*K downregulation in training-induced AV node dysfunction**

To decipher how training-induced transcriptional dysregulation of ion channels translates into slowed AV node conduction, protein expression of key dysregulated transcripts was assessed using immunohistochemistry or western blot, techniques limited by the specificity of commercially sourced antibodies. Connexins are responsible for electrical coupling between myocytes and therefore are an important determinant of the conduction velocity of the action potential. Protein levels of connexin 4045, 46 and connexin 45,47 previously shown to affect AV node conduction, were tested by immunohistochemistry and western blot respectively and found to be unchanged by training status (**Online Figure V**).

 *I*Ca*,T,*  carried in large part by the CaV3.1 channel in the adult AV node, is a known contributor to the diastolic pacemaker current.35 However, protein levels of CaV3.1 were not significantly altered in the trained AV node (**Online Figure V).** Contrastingly,in the case of CaV1.2, consistent with reduced mRNA levels, there was a significant downregulation of protein in the compact node of trained mice (n=7) *versus* sedentary mice (n=6) (**Figure 3A**). In all preparations the anti-Cav1.2 antibody produced the classical membrane labelling pattern that was prominent in sedentary animals (**Figure 3A**, top left panel, white arrows). In trained animals the labelling was faint, diffused and frequently absent (**Figure 3A**, top right panel, white arrows). Aggregates of non-specific artefactual labelling were evident in all mouse preparations (**Figure 3A**, top panels, blue arrows) and fluorescence intensity measurements from these structures were excluded from the analysis (see Online Supplemental Methods for further details). A qualitative reduction in CaV1.2 protein was also evident from subsequent western blot analysis (cf. **Figure 7F**).

To test directly whether reduced CaV1.2 expression has consequences for *I*Ca,*L*, AV node cells were isolated from sedentary and trained animals by enzymatic dispersion and were analysed by patch-clamp in the whole-cell configuration. The density of *I*Ca,*L* was recorded in AV node cells from sedentary (n=8 cells from 8 mice) and trained (n=9 cells from 9 mice) mice. Currents recorded in the range −55 to 20 mV were normalised to cell capacitance to obtain current density. The mean cell capacitance was 26.7 pF (n=22) and 27.5 pF (n=24) in sedentary and trained mice, respectively (not significantly different, Student’s *t*-test, P>0.05). In **Figure 3B** sample current traces and mean current–voltage (IV) relationships are shown. The *I*Ca,*L* density was reduced in trained mice by 30% at the peak value (0 mV).

As a role of *I*f in AV node conduction is becoming increasingly evident (see **Discussion**) and we have previously demonstrated a role for *I*f remodelling in the trained sinus node,24, 25 next we investigated the downstream consequences of HCN4 transcriptional remodelling in the AV node. Immunolabelling revealed a significant downregulation of HCN4 protein in trained mice (n=6) *versus* sedentary mice (n=6), especially evident in the penetrating portion of the His bundle (**Figure 3C**). As expected, this was accompanied by a reduction in *I*f over a wide potential range in isolated AV node myocytes from trained mice (n=9 cells from 9 mice) compared to sedentary mice (n=8 cells from 8 mice) (**Figure 3D**). Given these findings, the role of *I*f in AV node conduction was further assessed *in vivo* by blocking *I*f by administration of ivabradine (S16257-2; 6 mg kg−1), which dose-dependently reduces heart rate in humans48 and animals.49 Absolute PR intervals before and after block of *I*f in anaesthetised mice are shown in **Online Figure VI.** Intriguingly, block of *I*f abolished the difference in PR interval in trained (n=7) *versus* sedentary (n=7) mice, consistent with a training-induced downregulation in HCN channels being involved with or responsible for the difference, and further evidence for an important functional role for *I*f in AV conduction.

Next, the functional consequences of the change in expression of mRNAs coding for K+ channels presented in **Figure 2I** were assessed by patch clamp experiments in a separate cohort of swim-trained animals (n=5) and sedentary control (n=5) animals. A well-known K+ current in the mouse heart is the rapid delayed rectifier K+ current *I*K,r carried by the ERG1 channel. *I*K,r was recorded in AV node cells during and after depolarizing voltage clamp pulses as E-4031-sensitive current (E-4031 is a specific blocker of *I*K,r). Typical current recordings from AV node cells isolated from sedentary and exercise trained mice are shown in **Figure 4A**. Under control conditions, currents were smaller in exercise trained mice (**Figure 4A**). 1 μM E-4031 was then applied to block *I*K,r. E-4031 blocked some but not all current during the depolarizing pulses and **Figure 4A** shows both the E-41031-insensitive and -sensitive currents. Whereas ERG is responsible for the E-41031-sensitive current, other K+ channels are presumably responsible for the E-41031-insensitive current; both currents were smaller in exercise-trained mice (**Figure 4A**). Mean data are shown as IV relationships in **Figure 4B,C**. Current at the end of the depolarizing pulses (**Figure 4B**) and the peak tail current after the pulses (**Figure 4C**) was measured. Because *I*K,r is inactivated during depolarizing pulses (following activation) and this inactivation is rapidly removed on repolarization after the pulses, tail current is arguably the better measure of *I*K,r. **Figure 4B,C** shows current under control conditions as well as the E-4031-insensitive and sensitive currents and **Figure 4D,E** compares E-4031-insensitive and sensitive currents in sedentary and exercise trained mice. Both are smaller in exercise trained mice. The best measure of *I*K,r (E-4031-sensitive tail current in **Figure 4E**) suggests that *I*K,r is reduced by ~50% in exercise trained mice and this is consistent with the apparent trend towards downregulation of ERG mRNA (**Figure 2I**). However the difference did not reach statistical significance. Another well-known K+ current in the heart is the transient outward K+ current. It was measured in AV node cells as the 10 μM 4-AP-sensitive current during depolarizing pulses, but the current was undetectable both in sedentary and exercise trained mice (data not shown).

**Training-induced reduction in spontaneous firing of isolated AV node myocytes**

The combined impact of training-induced reductions in *I*Ca,*L* (**Figure 3B)** *I*f (**Figure 3D)** and *I*K (**Figure 4**) on the spontaneous action potential were tested in AV node myocytes isolated from sedentary and trained animals. Representative recordings from n=5 sedentary mice and n=5 trained mice are shown in **Figure 5A (**left panel) and a sample segment of an action potential recording with time- and voltage-hallmarks used for analysis is given in the right panel. As given in **Figure 5B**, the spontaneous beating rate of isolated AV node myocytes isolated from trained mice was significantly lower than that of sedentary mice, consistent with a reduction in *I*Ca,*L* and *I*f (**Figure 3B,D)**. Accordingly, the slope of both the linear and exponential phases of diastolic depolarization (SLDD and SEDD) was also significantly reduced in myocytes isolated from trained animals (Figure 5B). There were no discernible differences in the maximum diastolic potential, and action potential threshold, upstroke velocity, amplitude and duration (**Figure 5B)**. The predominant training-induced change to the action potential was the slowing of diastolic depolarization and a longer cycle length - likely explained by *I*Ca,*L* and *I*f reduction. As the observed changes in *I*K did not impact the maximum diastolic potential or action potential duration in trained *versus* sedentary AV node myocytes (**Figure 5B**) and previous studies in mice failed to observe alteration of the PR interval on *I*K,r suppression with ERG silencing50 or dofetilide treatment51 the study then focused on the training-induced reduction in *I*Ca,*L* and *I*f. To test whether the changes in *I*Ca,*L* and *I*f density could account for training-induced slowing of conduction through the AV node, the observed changes in *I*Ca,*L* and *I*f density (**Figure 3B,D**) were incorporated into a one-dimensional computational model of the AV node (**Figure 5C**). To simulate the effect of athletic training, the conductance of *I*fin the model was reduced by 60% and the conductance of *I*Ca,*L* in the model was reduced by 40%. The simulated I-Vrelationships of *I*Ca,*L* and *I*fare shown in right panels in **Figure 5C**. The model predicted a 25% reduction in the conduction velocity of the trained AV node action potential (7.1 cm/s) relative to the sedentary one (9.5 cm/s, **Figure 5C, left panel**).

From these combined *in vivo* (**Figure 2A-D, Online Figure VI**)*, ex vivo* (**Figures 2E-I**, **3, 4 and 5A,B**) and *in silico* (**Figure 5C**) approaches it is concluded that ion channel remodelling of the trained AV node is a primary mechanism underlying first degree AV block in swim-trained mice.

**Mechanisms controlling transcriptional remodelling of Cav1.2 and HCN4 in the trained AV node**

What regulates the transcriptional response of the trained AV node, in particular of CaV1.2 and HCN4? Our previous work has demonstrated that, in the trained sinus node, interplay of miR-423-5p and the transcription factor NKX2.5 controls mRNA expression of HCN4 and sinus bradycardia.24 Here we investigated, for the first time, the involvement of miRs as potential regulators of ion channel transcription within the AV node. Expression of 750 rodent miRs (and 6 reference transcripts) in the AV node was determined using a TaqMan Array system. Among the 750 miRs measured, 33 miRs were differentially expressed between sedentary (n=7) and trained (n=8) mice, given by the red circles in **Figure 6A** (values given in **Online Table S4**). Interestingly, only 2 out of 31 differentially expressed miRs were downregulated whereas the rest were upregulated by training (**Figure 6A,** red circles). These data are indicative of a repressive miR signature - a potential explanation for the diffuse training-induced downregulation of key pacemaking transcripts observed in **Figure 2I**. As 3′ untranslated region (3′UTR) targeting is acknowledged to be a primary method of miR-based gene silencing,52 significantly dysregulated miRs were screened by a dual luciferase assay to identify miRs that directly target the 3′ untranslated region (3′UTR) of CaV1.2 (**Figure 6B)** and HCN4 (**Figure 6C).** In this experimentrecombinant plasmids in which a luciferase coding sequence was cloned downstream of the **(i)** CaV1.2 3′UTR (split into three contiguous constructs A-C), **(ii)** HCN4 3′UTR and **(iii)** control vector without a 3′UTR (control) were co-transfected individually with 33 (differentially regulated) individual miRs in H9C2 cells. Firefly luciferase was measured and normalised to Renilla luciferase in duplicate experiments in independent batches of cells (**Figure 6B,C**). Based on this initial screen, many of the dysregulated miRs tested appeared to suppress luciferase activity linked to the CaV1.2 and HCN4 3′UTRs relative to control. However, of the 33 miRs tested, 14 miRs significantly decreased luciferase activity linked to the CaV1.2 3′UTR whereas five decreased the luciferase activity linked to the HCN4 3′UTR. Of these miRs, miR-215-5p, miR-211-5p, miR-335-3p, miR-380-5p, miR-1959 and miR-432 were selected for further study as: **(i)** they showed the most obviously inhibitory effects, i.e. >40% reduction in the normalised luciferase signal *versus* control and **(ii)** based on computational predictions by the algorithms Targetscan,53 RNA2254 and Sfold,55 consensus binding sites for these miRs were identified in the 3′UTR of CaV1.2 and HCN4 (**Online Figure VII)**. The effect of overexpression of these miRs on the CaV1.2/HCN4 3′UTR linked luciferase expression (**Online Figure VIIA-B,** based on an n=5 replicates in independent batches of cells) demonstrated that miR-215-5p, miR-211, miR-1959 and miR-432 repress the expression of an exogenous reporter gene with a CaV1.2 3′UTR (**Online Figure VIIA**) whereas miR-211-5p and miR-432 repress HCN4 3′UTR reporter expression (**Online Figure VIIB**).

A series of *in vitro* experiments were conducted to select miRs for *in vivo* targeting: first, site directed mutagenesis was conducted to verify specificity of predicted miR binding sites on the 3′UTR of CaV1.2 and HCN4 (wild-type and mutated sequences given in **Online Figure VIID**). In this experiment wild type and mutated 3′UTR sequences were co-transfected separately with selected miRs and compared to a control vector without a 3′UTR (control). Firefly luciferase was measured and normalised to Renilla luciferase in duplicate experiments in independent batches of cells. Of all miRs tested, only mutations in the binding sequence for miR-211-5p rescued the reduction in the bioluminescent signal for CaV1.2 (**Figure 6D**), revealing a potential direct binding region for Cav1.2 targeting by this miR. In the case of HCN4 (**Figure 6E**), mutations in predicted binding sites for miR-211-5p and miR-432 but not miR-335-3p rescued the reduction in the bioluminescent signal for HCN4 indicating two direct binding sites.

Having identified and confirmed functional bindings sites for miR-211-5p and miR-432 we tested whether endogenous CaV1.2/HCN4 expression can be modulated by these miRs. As primary AV node myocytes are not amenable to cell culture, we used embryonic stem-cell (ES) derived myocytes that natively express CaV1.2 and HCN4 transcripts.56 Parallel studies confirmed superior recruitment of HCN4 transcriptional machinery in these cells compared to other relevant cellular systems (HL-1 and neonatal rat ventricular cardiomyocytes tested; data not shown). miR mimics corresponding to miR-211-5p or miR-432 were introduced into embryonic stem cell-derived myocytes and overexpression after 24 h confirmed by qPCR. HCN4 transcript expression was suppressed by both miRs whereas Cav1.2 mRNA levels were only reduced by miR-211-5p overexpression (**Figure 6F**). Protein expression of HCN4 was also reduced by miR-432 overexpression (**Online Figure VIII**); western blotting for CaV1.2 protein was technically unfeasible due to diffuse non-specific antibody binding. Nevertheless, based on the combined approaches described above it is concluded that miR-211-5p (increased by 8-fold in the trained AV node) and miR-432 (increased by 70-fold in the trained AV node) are likely transcriptional regulators of Cav1.2 and HCN4 expression. Interestingly, these miRs demonstrated a trend towards upregulation in AV node biopsies from trained horses (n=5) compared to sedentary horses (n=5) (**Online Figure IX**).

**Reversibility of training-induced AV node remodelling with anti-miRs or detraining**

In pilot investigations, the impact of combined inhibition of miR-432 and miR-211-5p on training-induced PR interval prolongation was assessed in mice. The prolongation in the PR interval was evident by 21 days of swim-training, and at this time point, miRs were knocked down *in vivo* in swim trained mice (n=6) via 3 systemic (intraperitoneal) injections of 25 mg/kg body weight cholesterol-conjugated antisense oligonucleotides (antimiRs). AntimiR 211-5p and antimiR-432 were custom designed to include a 2′-O-methyl (2′-OMe) backbone linked by phosphodiester and phosphothiorate bonds conjugated to a 5′ cholesterol group. These chemical modifications have been recently optimised to give superior potency, efficacy and *in vivo* stability for miR targeting in the heart, resulting in a dramatic suppression of miRs one-week post administration.57 Littermate cohorts of sedentary (n=6) and trained mice (n=6) received an equivalent dose of control antimiR (oligonucelotides against *C. elegans* miR-39-5p that do not target mammalian sequences58). AntimiRs treatment did not impact overt health status or ability of animals to complete the training regimen. One week after the first administration (Day 28), a significant reduction in AV node levels of miR-432 in antimiR-treated animals was confirmed by qPCR; however, only modest suppression of miR-211-5p was achieved (**Figure 7A**). Unexpectedly, as given in **Figure 7B,** swim-trained mice that received antimiRs presented with a significant reduction in heart rate compared to swim-trained and sedentary mice that received control antimiRs. Despite this reduction in heart rate, trained mice that received the antimiRs did not develop a training induced prolongation of the PR interval observed in trained mice that received control antimiR (**Figure 7C**). To dissociate changes in the PR interval from the alterations to baseline heart rate, the PR interval was measured during fixed S1-S1 pacing starting at 120 ms. At this cycle length, trained animals receiving control antimiR demonstrated Wenckebach conduction. Therefore S1-S1 pacing at 130 ms was attempted. **Figure 7D** shows that at a fixed pacing cycle length of 130 ms, the PR interval was longer in trained animals and that this effect was abolished by antimiRs targeting CaV1.2 and HCN4. Representative recordings obtained during *in vivo* programmed electrical stimulation demonstrating the effect of systemic antimiRs suppression on the PR interval under fixed S1S1 pacing are given in **Figure 7E**. To test whether reversal of PR prolongation in the antimiRs-treated trained animals was accompanied by a restoration of CaV1.2 and HCN4 to pretraining levels, AV node biopsies (n=3/group/protein) were harvested for western blotting. As shown by representative western blots (**Figure 7F,G**), there was a reduction in Cav1.2 (**Figure 7F,** right panel) and especially HCN4 levels (**Figure 7G,** right panel) in the trained AV node accompanied by restoration in the antimiRs-treated group. In both cases there was a heterogeneity in the individual response to antimiR treatment but this was not investigated further. Nevertheless, in both instances there was no longer a significant difference in the expression levels of CaV1.2 and HCN4 between sedentary animals and trained animals receiving antimiRs. Finally, the impact of detraining was investigated. As given in **Figure 7H,I**, 12 weeks of detraining restored training-induced sinus bradycardia and PR prolongation *in vivo*. Intriguingly, only HCN4 expression was de-repressed in detrained animals (**Figure 7K**); the reduction in CaV1.2 persisted (**Figure 7J**). It is concluded that either miR suppression or detraining rescues training-induced AV node dysfunction by (at least) partial reversal of electrical remodelling.

**DISCUSSION**

This study shows that dysfunction of the AV node in response to sustained endurance exercise cannot be fully attributed to changes in autonomic tone as commonly accepted, and is likely to be due in part to transcriptional remodelling of ion channels. Based on data collected in two animal models of endurance exercise, we ascribe particular importance to a downregulation of CaV1.2 and HCN4 and a consequent decrease in the density of *I*Ca,L and *I*f as a new molecular mechanism underlying AV block in athletes. We also identify miR-211-5p and miR-432 as potential new regulators of electrical remodelling within the trained AV node.

**Horse as a high-fidelity model for training-induced rhythm disturbances**

To our knowledge, this is the first exploration of training-induced arrhythmia in a large animal model. To date, all studies concerning the effect of long-term exercise on cardiac function have been conducted in human athletes (primarily in non-invasive studies) or, when mechanisms such as fibrosis, inflammation and vagal influence are studied, in rodents.24, 25, 41, 42, 59 The cardiac adaptation to long-term physical training in racehorses is identical to the changes observed in human athletes leading to the development of comparable phenotypic characteristics of the ‘Athlete’s Heart’ such as ventricular hypertrophy, and reduced fractional shortening and diastolic function after exercise.26, 27, 60 Sinus arrhythmia, sinus node exit block, wandering pacemaker, and AV block have been reported in horses.61 Remarkably, second-degree AV block is the most common arrhythmia, reported to occur in up to 40% of horses.61 These characteristics make the racehorse uniquely suited for modelling the effect of sustained exercise on the cardiac conduction system. Correlations between detraining time, the heart rate and PR interval were studied (**Figure 1H-I**). Exponential analysis showed that the rate of reversibility of sinus node remodelling (τ = 10.3 months) was slower than that of AV node remodelling (PR interval, τ = 2.5 months). Literature on reversibility of AV node dysfunction in human athletes is sparse but our findings in both racehorses (**Figure 1H**) and in mice (**Figure 7I**) corroborate a recent study reporting that prolongation of the PR interval induced by 12 weeks of moderate-intensity aerobic exercise in postmenopausal women was lost after five months of detraining.62

**Role of intrinsic *versus* autonomic mechanisms underlying AV block in athletes**

Autonomic innervation of the AV junction modulates conduction of the action potential, the sympathetic nerves facilitating and parasympathetic nerves impeding conduction. On the basis of heart rate variability measurements, AV block in athletes is currently attributed to increased vagal tone,22 even though many studies in humans and animal models show no evidence of high vagal tone (reviewedby D’Souza and Boyett23, 63, 64). Furthermore, our recent work has shown from an analysis of the underlying biophysics of pacemaking that heart rate variability is primarily determined (in an exponential-like manner) by heart rate, and the increase in heart rate variability in athletes is attributed to the resting bradycardia rather than any increase in vagal tone.65The results of this study add further weight to the evidence against high vagal tone in training-induced AV node dysfunction. In horses (**Figure 1K**) and mice (**Figure 2A**), the PR interval was prolonged in the trained animals both under baseline conditions and after administration of atropine and [propranolol](https://www.sciencedirect.com/topics/medicine-and-dentistry/propranolol) to block the autonomic nervous system. These data corroborate previous observations in human athletes by Stein *et al.*10 in which the Wenckebach cycle length and anterograde AVERP were prolonged in athletes, regardless of the presence of autonomic blockade. Similarly, in a study investigating second-degree AV block in horses, Yamaya *et al.*66 reported that slower AV conduction, prolonged Wenckebach conduction and higher refractoriness in horses with AV block (*versus* horses without AV block) was still maintained under complete autonomic block. Furthermore, experiments in the isolated, denervated mouse AV node demonstrated that conduction slowing was intrinsic to the node (**Figure 2G**), further reiterated in the observed slowing of the spontaneous cycle length in isolated AV node myocytes from trained *versus* sedentary mice (**Figure 5A-B**). From all these lines of evidence - ranging from the intact animal(s) to the isolated myocyte - we propose a predominant role for intrinsic electrical remodelling of the AV node in explaining first-degree AV block in athletes.

**Ion channel remodelling in the trained AV node**

This study presents for the first time a comprehensive transcriptional and electrophysiological analysis of the known key players underlying action potential generation and conduction in the mouse AV node and its dysregulation in the setting of prolonged exercise training (**Figure 2I; Online Table S2**). To avoid contamination of the AV node tissue samples by the neighbouring atrial and connective tissues, laser capture microdissection (**Figure 2H**) was used; our AV node transcriptional profile provides a benchmark for other investigators studying mouse AV node physiology and transcriptional remodelling. The striking and selective downregulation of ion channel and related transcripts in the AV node but not in the surrounding right atrial muscle (**Online** **Figure IV**), led us to investigate the role of miRs in this process, given their capacity to regulate the levels of multiple genes simultaneously. Accordingly, in this study we demonstrate, for the first time, that AV node ion channels are under the control of the miR network. As primary mouse AV node myocytes are not amenable to culture, mouse ES-derived myocytes that natively express Cav1.2 and HCN4 transcripts were used in this study as an intermediate step in the selection of miRs for *in vivo* targeting (based on the efficacy of miR-mediated knockdown). However, under standard culture conditions these cells appeared quiescent, precluding the assessment of miR-mediated electrophysiological alterations. Nevertheless, we identified and validated direct binding regions for miR-211-5p/miR-432 mediated targeting of CaV1.2 and HCN4 (**Figure 6**) and on the basis of these findings tested the therapeutic efficacy of targeting noncoding miRs to rescue AV node dysfunction *in vivo*. Our study provides first proof-of-concept that *in vivo* suppression of miR-211-5p and miR-432 rescues the training induced prolongation of the PR interval with restoration of HCN4 protein to pre-training levels (**Figure 7**). However, given the unexplained preferential suppression of miR-432 *versus* miR-211-5p and a lower heart rate in antimiRs-treated animals in this pilot miR study, we conclude that major challenges of specificity and delivery remain for development of oligonucleotide-based therapeutics for AV block. Adeno-associated virus-based antimiRs gene delivery engineered to enhance transduction specificity to nodal myocytes holds promise in this regard.

In this work we report that endurance exercise in mice results in diminished *I*Ca,L *, I*f and *I*K density. In particular, we associate remodelling of the inward currents, *I*Ca,*L* and*I*f, with the decrease in the spontaneous rate and slowed diastolic depolarisation seen in isolated AV node myocytes from trained *versus* sedentary mice (**Figure 5A-B**).

While this study is the first to associate Cav1.2 downregulation (and consequently *I*Ca,L downregulation) with exercise-induced AV block (**Figure 3A,H**), the relationship between AV block and L-type Ca2+ channel remodelling is not new and has been demonstrated in both acquired and inherited pathological states. For example, mutations in the L-type Ca2+ channel have been identified in humans with syndromic AV block67 and congenital AV block has been associated with maternal antibodies targeting the L-type Ca2+ channel in infants.68 We have previously reported that downregulation of L-type Ca2+ channels contributes to AV node dysfunction following pulmonary hypertension.38 Given the role of *I*Ca,L in underlying the slow phase 0 upstroke of the action potential in the slow conducting pathway of the AV node,30 these findings are expected. A number of commercially available antibodies were tested to assess CaV1.2 remodelling in mice – all of which produced labelling artefact that could be excluded by automated image analysis from immunolabelling. Future mass spectrometry-based analysis may be required to arrive at a definitive conclusion but the small size (~30 μg of protein/mouse AV node) precluded this assessment in the present study. Nevertheless, the overall trend in CaV1.2 downregulation remained consistent between techniques, experiments and species (**Figures 1R-S, 3A** and **7F**).

In contrast to *I*Ca,L, the link between *I*f downregulation (**Figure 3C-D**) and AV block is less well established. HCN channels and *I*f have mainly been considered in relation to sinus node pacemaking, but there is now substantial evidence that *I*f may play a significant role in AV node conduction: The results of this study (**Table S2**) and others from our laboratory37, 38 have demonstrated high levels of HCN4 in the AV node. HCN4 mutations have been associated with AV block in humans69 and, in the same vein, Baruscotti *et al*.34 demonstrated thatinducible cardiac-specific knockout of HCN4 in mice is lethal because of the development of complete AV block. Consistent with the work by Baruscotti *et al*.,34 it has been shown that mice carrying conditional time-controlled and heart specific genetic silencing of *I*f present with a high incidence of type 1 and 2 second-degree AV block, an observation which correlates with strong reduction of automaticity of AV node myocytes recorded in this model.39 In addition, PR prolongation induced by the *I*f blocker ivabradine (**Online Figure VI**) observed in this study is in agreement with previous mouse studies in which *I*f block reduced AV node automaticity.36, 39, 70 Similarly, the *I*f blocking agent, zatebradine, has been shown to increase the A-H interval, AVERP, and Wenckebach cycle length in humans.71 We have also recently shown that in young rats, *I*f block significantly prolongs the A-H interval, AVERP and Wenckebach cycle length.37 We posit that downregulation of *I*f may slow AV node conduction by decreasing cell excitability. In this work, reversal of HCN4 but not CaV1.2 transcriptional remodelling with detraining (**Figure 7J-K**) coupled with the findings that acute *I*f block abolishes training-induced PR prolongation (**Online Figure VI**) suggest that HCN remodelling may be the predominant effector of AV block in trained mice. Detailed pharmacological dissection of the individual contribution of these channels towards the observed phenotype was not within the study aims but merits further investigation.

**Concluding remarks**

In conclusion, this study has uncovered fundamental ionic and transcriptional changes that set the electrophysiological milieu of the trained AV node. These findings provide new insight into the molecular mechanisms underlying a common rhythm disturbance in athletes. Follow-on studies investigating AV node-specific delivery of miR-211-5p and miR-432 may be an important first step towards the development of small molecule therapies for AV block seen in some veteran athletes.

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**DISCLOSURES**

None

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**FIGURE LEGENDS**

**Figure 1. AV block and AV node intrinsic electrical remodelling in racehorses. A-D,** Representative ECG recordings obtained during 24 h radiotelemetry in sedentary (n=6) and trained (n=13) horses. PR intervals (ms) are highlighted in red. **A**, Typical sinus rhythm in a sedentary horse. **B**, Example of type 1 second-degree AV block in a trained horse characterised by irregular PR intervals preceding a dropped QRS complex. **C**,Example of ‘Wenckebach phenomenon’ observed in trained horses characterised by lengthening PR intervals preceding a dropped QRS complex. **D**, Example of second-degree AV block in a trained racehorse that does not meet the criteria for type 1 second-degree AV block. **E-G,** Heart rate,PR interval and incidence of second-degree AV block in sedentary (n=6) and trained (n=13) horses (\*P<0.05, Student’s *t* test; statistical significance for incidence of second-degree block tested using Mann-Whitney test). **H,** Relationship between heart rate and duration of detraining. **I**, Relationship between PR interval and duration of detraining. Data in H-I fit by exponential decay curves; best fit curves, R2 and τ values shown. Red circles denote trained horses and blue circles denote detrained horses. **J**, PR intervals measured in 10 beat epochs corresponding to the minimum and maximum heart rate from 24 h radiotelemetry recordings in sedentary (n=5) and trained (n=5) horses. **K**, PR intervals measured at baseline and on complete pharmacological autonomic blockade in sedentary (n=6 at baseline and n=4 for autonomic block) and trained (n=5 at baseline and n=5 for autonomic block) horses (\*P<0.05, Mann-Whitney test). **L**, Masson’s trichrome staining of horse AV node demonstrating the location of the compact node (red square). Right panel shows the compact node at a magnified scale. **M**, Representative HCN4 (green signal; top panels) and Cav1.2 (red signal; lower panels) immunostaining in the compact AV node from sedentary and trained horses. Scale bar = 10 μm. **N**, Signal intensity of HCN4 immunolabelling in sedentary (n= 3 slides/horse from 7 horses) and trained (n= 3 slides/horse from 7 horses) horse AV nodes. Data pooled from two sets of independent experiments and protein expression is normalised to sedentary value (set at 100%) (P value shown, Student’s *t* test). **O**, Representative HCN4 western blot from AV node biopsies from sedentary (n=4) and trained (n=5) horses. Corresponding stain-free total protein membrane used for quantification shown in lower panel. **P**, HCN4 protein expression from western blot (n=5/5, P value shown, Student’s *t* test)**. Q**, Signal intensity of CaV1.2 immunostaining in sedentary (n=7) and trained (n=7) horse AV node. Data pooled from two sets of independent experiments and protein expression is normalised to sedentary value (set at 100%) (\*P<0.05, Student’s *t* test). **R**, Representative CaV1.2 western blot from AV node biopsies from sedentary (n=4) and trained (n=4) horses. Corresponding stain-free total protein membrane used for quantification shown in lower panel. **S**, CaV1.2 protein expression from western blot (n=5/5, \*P<0.05, Student’s *t* test)**.**

**Figure 2:** **Long-term training induces AV node dysfunction and transcriptomic remodelling**. **A**, PR interval obtained from anaesthetised sedentary and trained mice at baseline (n=6 sedentary/11 trained) and on complete autonomic blockade (n=14 sedentary /13 trained), at cessation of 20 weeks swim training (\*P<0.05, 2-way ANOVA with Sidak’s multiple comparisons test). **B**, Pacing cycle lengths at which Wenckebach conduction (WCL) occurred and AVERP at 100 ms pacing frequency in sedentary (n=14 for WCL and n =13 for AVERP) and trained (n=11) mice (\*P<0.05, Student’s *t* test). **C**, Representative recordings obtained during *in vivo* S1-S1 pacing in sedentary (black trace, pacing cycle length 74 ms) and trained (red trace, pacing cycle length 106 ms) mice illustrating the prolongation of the Wenckebach cycle length in trained mice. S, stimulus artifact; V, ventricular deflection. S-V interval is given in ms. **D**, PR interval during *in vivo* S1-S1 pacing at fixed cycle lengths of 100 and 120 ms (n=13 sedentary and 10 trained mice; \*P<0.05, 2-way ANOVA with Sidak’s multiple comparisons test). **E**, Example of AV node preparation used for extracellular action potential recording. Red circles indicate electrode positions. CS, coronary sinus; FO, fossa ovalis; IVC, inferior vena cava; RAA, right atrial appendage; SVC, superior vena cava. Scale bar = 2 mm. **F**, Spontaneous cycle length of sedentary (n=8) and trained (n=7) AV node preparations. \*P<0.05, Student’s *t* test. **G** (left panel), Representative spontaneous extracellular potential recordings from sedentary (black trace) and trained (red trace) preparations demonstrating atrial (A) and His (H) signals (marked by vertical lines). **G** (right panel), Spontaneous and paced (at a 150 ms cycle length) A-H intervals obtained from extracellular potential recordings from sedentary (n=8 for spontaneous and n=4 for paced) and trained (n=7 for spontaneous and n=4 for paced) mice. \*P<0.05, 2-way ANOVA with Sidak’s multiple comparisons test). **H (top panel)**, Masson’s trichrome staining of a 20 μm cryosection of the mouse AV node, demonstrating the location of the AV conduction axis (red square). Scale bar = 500 μm. Inset shows the compact node (circled) at a magnified scale as a compact, ovoid bundle of loosely packed cells distinguishable from the inferior ventricular myocardium (stained purple) by the central fibrous body, a band of connective tissue (stained royal blue). Scale bar = 100 μm. **H (lower panel)**, Laser capture micro-dissection of the AV node tissue from a mouse heart.Left lower panel, HCN4-immunolabelled mouse AV junction corresponding to the boxed area in the lower middle panel. Lower middle panel, 10 μm cryosectioned AV junction of a mouse heart stained with cresyl violet and mounted on a membrane slide for laser capture microdissection. Right panel, the same slide after laser capture microdissection. Scale bars = 200 μm. **I,** mRNA expression of Ca2+ channels, Ca2+ handling molecules, HCN channels, K+ channels and connexins in laser-capture microdissected AV node samples from sedentary (n=5-7) and trained mice (n=5-7) normalised to the expression of *Hprt* and *Pgk1* (\*P<0.05, limma test followed by Benjamini-Hochberg false discovery rate correction). See tables S2 and S3 for n numbers for respective transcripts.

**Figure 3: Reduced *I*Ca,L and *I*f density in trained mice. A (top panel),** Representative Cav1.2 (red signal) immunostaining in the compact AV node from sedentary and trained mice. Scale bar = 10 μm. White arrows denote specific membrane labelling of Cav1.2. Blue arrows indicate examples of labelling artefact that was not included in the image analysis. **A (lower panel)** Summarised signal intensity of Cav1.2 immunolabelling in sedentary (n=6) and trained (n=7) mouse compact AV node (significance assessed by Student’s *t* test). **B**, Representative examples of *I*Ca,L from single AV node cells isolated from sedentary (left) and trained (right) mice. The inset shows the voltage clamp protocol used. Bottom, *I*Ca,L IV relationships recorded from sedentary (n= 11 cells from 5 mice) and trained (n= 13 cells from 6) mice. Significance was assessed using a mixed effects linear model on log-transformed data. Sidak’s multiple comparisons test was applied for multiple testing correction. P values shown were computed on the geometric mean; density of *I*Ca,*L* is shown. **C (top panel)**, Representative HCN4 (green signal) immunolabelling in the penetrating bundle from sedentary and trained mice. The nodal cells lie within the dashed white lines. Scale bar = 200 μm. **C (lower panel)**, Summarised signal intensity of HCN4 immunolabelling in sedentary and trained mouse penetrating bundle (n=6/ group, significance assessed by Student’s *t* test). **D,** Representative examples of *I*f from single AV cells isolated from sedentary (left) and trained (right) mice. The inset shows the voltage clamp protocol used. Bottom, *I*f IV relationships recorded from sedentary (n= 9 cells from 4 mice) and trained (n= 8 cells from 4 mice) mice. Significance was assessed using a mixed effects linear model on log-transformed data. Sidak’s multiple comparisons test was applied for multiple testing correction. P values shown were computed on the geometric mean; density of *I*f is shown.

**Figure 4**. **Training effect on *I*K,r density. A**, Typical examples of ionic currents in AV node cells isolated from sedentary (top panels) and exercise trained (bottom panels) animals under control conditions and after perfusion of 1µM E-4031. E-4031 sensitive current (calculated as ‘Control’ minus ‘E-4031-insensitive’ current) in sedentary and trained cells is shown in the right panel. **B,** IV relationships of *I*K in AV node cells of sedentary (left, n=15 cells from 4 mice) and exercise trained (right, n=17 cells from 5 mice) animals measured at the end of the depolarizing step before (black circles) and after (blue circles) 1 µM E-4031 application. In red is shown the E-4031-sensitive current density. **C**, same as panel B, but current density was measured just after the pulse (peak tail current). **D**, IV curve for E-4031-insensitive (left, blue circles) and E-4031-sensitive (right, red circles) current measured at the end of the depolarizing step in sedentary (filled symbols) and exercise trained (empty symbols) animals. **E**, same as in D but with peak tail current values. Significance was assessed using a mixed effects linear model on log-transformed data. Sidak’s multiple comparisons test was applied for multiple testing correction. P values shown were computed on the geometric mean.

**Figure 5. Training-induced reduction in spontaneous firing of AV node myocytes due to *I*Ca,L and *I*f underliesslowing of AV node conduction. A (left panel),** Representative action potential recordings from AV node myocytes isolated from sedentary (from n = 9 cells from 4 mice) and trained (from n= 11 cells from 5 mice) animals. The dotted line indicates 0 mV. **A (right panel)**, Action potential recording with time- and voltage-hallmarks used for analysis. **B**, Table summarising AV node action potential parameters recorded from sedentary and trained mice. MDP, maximum diastolic potential; SLDD, slope of the linear phase of diastolic depolarisation; SEDD, slope of the exponential phase of diastolic depolarisation; dV/dt, action potential upstroke velocity; APD, action potential duration. Significance was assessed using a mixed effects linear model. Sidak’s multiple comparisons test was applied for multiple testing correction. P value for SLDD was computed on the geometric mean.**C**, Computational simulation of the effect of the observed changes in *I*Ca,L and *I*f density on AV node conduction. Top, diagram of the one-dimensional model used to simulate conduction through the AV node. The left end of the string of cells was stimulated, and the action potential was recorded at two sites separated by 2 mm in the centre of the string as the action potential propagated along the string. The interval between the two action potentials recorded is longer the slower the conduction velocity. Left, AV node action potentials recorded at the two sites in simulations of the AV node of sedentary and trained mice. The interval between the two action potentials is longer in the case of the simulation of the AV node of the trained mouse; this corresponds to a slower conduction velocity. Right, simulated IVrelationships of *I*Ca,L and *I*fin sedentary and trained AV node cells (based on the data in Figure 3B,D).

**Figure 6: miRs repress CaV1.2/HCN4 expression by 3′UTR targeting.** **A**, Expression of 750 miRs in the AV node measured using TaqMan TLDA cards in the AV node of mice. Ratio of miR expression in trained mice (n=8) to expression in sedentary mice (n=7) shown on a logarithmic scale. Red circles indicate significant varying miRs (P<0.05, Limma test with Benjamini-Hochberg false discovery rate correction). **B and C**, Luciferase reporter assay screen for candidate miRs that target the 3′UTR of Cav1.2 (B) and HCN4 (C). Ratio of Luciferase to Renilla activity was measured in H9C2 cells 24 h after miR cotransfection and compared to control reporter plasmid (no 3′UTR) in H9C2 cells. Error bars indicate range of duplicates in two independent batches of cells. **D and E,** Luciferase/Renilla activity ofwild-type or mutant3′UTR of CaV1.2 (D) and HCN4 (E) after 24 h miR co-transfection (n=3 replicates in three independent batches of cells; \*P<0.05 *versus* control, one-way ANOVA with Holm-Sidak’s multiple comparisons test). **F**, Effect of miR overexpression on CaV1.2 and HCN4 (transcript levels measured 24 h after transfection in ES-derived myocytes). mRNA expression normalised to expression of *Ipo8* and *Hprt.* Data from n=3 independent batches of cells run in triplicate (\*P<0.05, one-way ANOVA with Dunnett’s multiple comparisons test).

**Figure 7: Reversal of training-induced PR interval prolongation by miR suppression or detraining.** **A**, Expression of miR-211-5p and miR-432 in the AV node of control or antimiR-treated sedentary and trained mice (n=6/6/6). Transcript expression is normalised to *Snord61* and *Snord95*. Significance assessed by Kruskal-Wallis test with Dunn’s multiple comparisons test. **B-D,** Mean heart rate (B), PR interval (C) and PR interval under a constant S1-S1 pacing cycle length of 130 ms (D) measured in anaesthetised antimiRs-treated sedentary and trained mice (n=6/6/6). Significance assessed by Kruskal-Wallis test with Dunn’s multiple comparisons test (**B**) or one-way ANOVA with Dunnett’s multiple comparisons test (**C-D**). **E**, Representative recordings obtained during *in vivo* S1-S1 pacing in sedentary (black trace, from n=6) trained (red trace, from n=6) mice illustrating the restoration of PR interval to sedentary values with antimiR-432 and antimiR-211-5p (blue trace, from n=6). Downward deflection indicates stimulus artefact; P and R waves labelled. **F** **(left panel)**, Representative CaV1.2 western blot using AV node biopsies from control or antimiR-treated sedentary and trained mice. Corresponding stain-free total protein membrane used for quantification shown in lower panel. **F (right panel)**, CaV1.2 protein expression (normalised to total protein) from western blot (n=3/3/3 run in duplicate). **G** **(left panel)**, Representative HCN4 western blot (normalised to total protein) using AV node biopsies from control or antimiR-treated sedentary and trained mice. Corresponding stain-free total protein membrane used for quantification shown in lower panel. **G (right panel)**, HCN4 protein expression from western blot (n=3/3/3 run in duplicate. \*P<0.05, one-way ANOVA with Holm-Sidak’s multiple comparisons test. **H and I,** Mean heart rate (H) and PR interval (I) obtained in conscious (n=8/8/8 for heart rate and 7/7/7 for PR interval) and anaesthetised (n=8/7/8 for both heart rate and PR interval) sedentary, trained and de-trained mice. **J and K,** mRNA expression ofCaV1.2 (J) and HCN4 (K) measured in AV node biopsies from sedentary (n=7), trained (n=7) and detrained (n=7) mice. Transcript expression normalised to *Ipo8*, *Hprt* and *Tbp1*. Significance for H-K assessed by one-way ANOVA with Tukey’s multiple comparisons test with the exception of the anaesthetised group in H where significance was assessed by Kruskal-Wallis test with Dunn’s multiple comparisons test.