**ONLINE DATA SUPPLEMENT**

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

**SUPPLEMENTAL METHODS**

**Horse model**

**Horses**

The study was approved by the local ethical committee at the Department of Veterinary Clinical Sciences, University of Copenhagen and the Danish Animal Experiments Inspectorate, and was performed in accordance with the European Commission Directive 86/609/EEC. Sedentary, trained and detrained Standardbred trotters stabled in equine facilities at the University of Copenhagen were studied; sedentary and trained groups had two and three castrated males (geldings) respectively whereas the remaining horses were mares (sedentary = 6 ± 0.57 years of age; trained = 6.2 ± 0.57 years of age; detrained = 7.4 ± 1.28 years of age, no significant age differences between groups, P>0.05, one-way ANOVA).

Out of 6 horses allocated to the sedentary group, 5 horses had never participated in a race whereas one horse had participated in one race over 4 years prior to enrolment in the study but had not trained since. Sedentary horses were allowed to walk in a pasture daily. All horses in the trained group were in full training for at least 3 consecutive years. The horses were ‘race fit’ at the time of enrolment in the study and had competed at professional races within the same month of purchase. The training regimes varied according to trainer-preferences, but in general involved exhaustive training 3-5 times a week. The training typically involved 1 h sessions of either long runs in heavy soil, interval training or sprint sessions. Trained horses were also subjected to ‘loosening’ exercise in a walker. Horses in the detrained group were retired racehorses that were housed in the equine facility at the University of Copenhagen and had been retired from training for varying durations (2–60 months). It was not possible to perform randomization, allocation concealment or blinding in horse studies as animals were procured based on their training history.

**ECG recording**

As a part of the clinical examination for enrolment in the study, all horses had a Holter ECG performed at rest in the stall over a 24 h period using a Holter unit with 2 separate channels and bipolar leads as previously described.1 Electrodes were placed in a modified base-apex lead and secured by adhesive foam pads. Based on clinical and echocardiographic examination, horses were considered clinically healthy with no apparent cardiovascular diseases. For data presented in Figure 1E-G, ECG recordings were obtained between the hours of 14:00-17:00 on a separate occasion within 2 weeks from enrolment.

**Autonomic block**

Horses were instrumented for Holter ECG recording as described above and the baseline ECG recorded for 15 min at 8:00 am. Complete autonomic blockade was achieved by simultaneous intravenous injection of propranolol 0.20 mg/kg followed by atropine 0.04 mg/kg. The ECG was continuously recorded for 1 h after atropine injection.

**Analysis of ECG data**

To compute the 24 h variation in heart rate and PR interval the RR intervals from the 24 h Holter recordings were exported to HRV Premium 3.1.0 software (Kubios) from which the average heart rate from 30 min bins over a 24 h period were obtained. PR intervals were manually measured from 10 consecutive beats from ECG segments corresponding to the minimal and maximal heart rate. For all other panels in Figure 1, ECG recordings were imported into LabChart 7 (v7.3.8, ADInstruments). Heart rate and PR intervals were measured over 100 consecutive beats.Beats that included an episode of AV block were not included in these measurements (as the PR interval could not be measured) and the next full waveform was substituted. ECG data were manually analysed for absent QRS complexes, which were categorized as second degree AV block (**Figure 1G**) without differentiating between Type I and Type II second degree AV block, because, generally, type II second-degree AV block is rare in athletes and difficult to diagnose, and its incidence is most commonly due to the misdiagnosis of type I second-degree AV block.2 In humans, transient sympathetic and parasympathetic surges can cause type I second-degree AV block to appear as type II second-degree AV block on ECG.3 Additionally, the infranodal dysfunction of type II second-degree AV block is typically accompanied by a widened QRS complex, which was not seen in any horse. Third degree AV block was not observed in any recording.

**Mouse model**

**Mice**

Care and use of laboratory animals conformed to the United Kingdom Animals (Scientific Procedures) Act 198, and European (86/609/CEE) regulations and guidelines. Ethical approval for all experimental procedures was granted by the University of Manchester. Eight-week-old male C57BL/6j mice (Harlan Laboratories; initial body weight, 20–25 g) were randomly assigned to either sedentary or trained groups. Only male mice were used as in our previous experience they are more amenable to swim training. Allocation concealment and blinding was not possible except in the case of echocardiography experiments due to the limited number of trained personnel available to perform techniques. Mice were housed five per cage in a temperature-controlled room (22°C) with a 12 h:12 h light:dark lighting regime and free access to food and water. No animals were excluded from analyses

**Anaesthesia**

For echocardiography, ECG recording and *in vivo* programmed electrical stimulation, anaesthesia was induced with 2% isoflurane in 100% O2 with a flow rate of 1 l /min and maintained with 1.5% isoflurane in 100% O2 with a flow rate of 1 l /min.

**Swim training**

Mice were subjected to a swimming programme described previously.4,5 Mice were swim-trained in a tank with a surface area of 32,500 cm2, depth of 35 cm and water temperature of 32 °C. In the first set of experiments, mice were trained for 10 min day−1 and the duration of exercise was extended in daily increments of 10 min until finally reaching 60 min. This duration of exercise was maintained for 20 weeks, which approximates to >10 years of endurance training in humans.6 In the second set of experiments, mice were trained for 60 min day−1 for 4 weeks. Both training protocols produced a prolongation of the PR interval evidenced by ECG recording as described below. In a further cohort of mice, after the last training session (4 weeks), mice were submitted to detraining for 12 weeks in which physical activity was restricted to the space of the cage.  All mice were able to complete the training protocol. Age- and weight-matched sedentary littermates served as controls for all experimental conditions and were handled daily.

**AntimiR treatment**

AntimiR design and dosing was based on previous studies where optimal chemistry for cardiac delivery and knockdown efficiency was determined.7 Sequences used were as follows:

miR-211-5p antimiR:  5′-Chol-\*A\*G\*GCAAAGGAUGACAAAGG\*G\*A\*A-3’

miR-432 AntimiR:  5′-Chol-\*C\*U\*GCCCACUGAUCUACUCCA\*A\*G\*A-3’

Control AntimiR:  5′-Chol-\*A\*A\*G GCAAGCUGACCCUGA A\*G\*U\* U-3'

where \* denotes a phosphothiorate and 5′-Chol-\*oligonuleotide denotes a 5′-cholesterol modification. AntimiRs were diluted in sterile saline and administered via intraperitoneal injection on three consecutive days (25 mg/kg body weight). Animals were randomly allocated to injection groups

**Echocardiography**

At the end of the training period, transthoracic echocardiography was conducted in sedentary and trained mice under anaesthesia (1.5% isoflurane in 100 O2 with a flow rate of 1 l /min) as described previously.8 Briefly, an Acuson Sequoia C256 ultrasound system fitted with a 14-MHz transducer (Siemens) was used to image the heart in the two-dimensional short-axis view. Left ventricular end diastolic diameter, left ventricular end systolic diameter, and diastolic posterior wall and interventricular septal thicknesses in diastole were assessed by M-mode echocardiography. Measurements were obtained using the leading-edge method over a minimum period of three cardiac cycles, with the researcher blinded to treatment groups.

**Unconscious ECG recordings**

At termination of the experiment, ECGs were recorded under isofluorane anaesthesia as described previously.4 In separate cohorts of mice, the effect on heart rate and the PR interval of (i) complete autonomic block using a combination of 0.5 mg kg−1 atropine and 1 mg kg−1 propranolol and (ii) 6 mg kg−1 ivabradine were determined as previously described.4

***In vivo* programmed electrical stimulation**

Animals were terminally anaesthetised (1.5% isoflurane in 100% O2 with a flow rate of 1 l /min) and ventilated with a respirator, following which the right atrium was accessed by thoracotomy and an EPR-800 catheter (Millar Instruments) introduced. Electrical stimulation was delivered via a DS3 isolated constant-current stimulator (Digitimer Ltd.) interfacing with a CED Micro3-1401 data acquisition system and controlled by Spike software (Cambridge Electronic Design). Pacing protocols were performed to measure sinus node recovery time, AV node effective refractory period and Wenckebach cycle length as given previously.910 Briefly, the threshold was determined using a fixed S1-S1 protocol with an interval 10 ms below the spontaneous cycle length. A square pulse of duration 2 ms was gradually increased from 0 and the minimum voltage required to capture the atrium was determined. The stimulating voltage was set at 0.5 V higher than the threshold and the output was kept constant for all subsequent pacing protocols. Sinus node recovery time (SNRT) was determined using a S1-S1 protocol with a cycle length of 100 ms, 120 ms and 130 ms. The time taken for the first spontaneous atrial beat after cessation of pacing was defined as the SNRT. Corrected SNRT (cSNRT) was calculated by subtracting the RR interval from the SNRT. Wenckebach cycle length was determined by pacing the atrium for 30 s, starting at a cycle length 10 ms below the spontaneous cycle length, and then a progressively shortened cycle length until Wenckebach conduction was seen between the atrium and the ventricle (Figure 2B). A further set of 30 s S1-S1 pacing protocols was undertaken starting at 11 ms longer than the cycle length at which Wenckebach conduction had occurred. If 1:1 atrial to ventricular conduction occurred the protocol was repeated with a 1 ms reduction in the cycle length of the S1-S1 protocol. The cycle length at which 1:1 atrial to ventricular conduction failed and Wenckebach conduction occurred using 1 ms reductions in cycle length was recorded as the Wenckebach cycle length. AVERP was determined using an S1-S2 protocol. There was an initial drive train of S1 beats (S1-S1, 100 ms) for 8 beats. The initial S1-S2 coupling interval was 100 ms. If the extrastimuli captured the atrium and was conducted to the ventricle the protocol was repeated with a 10 ms reduction in the S1-S2 coupling interval. This was repeated until AV conduction was lost. A new set of protocols was run again with the S1-S2 coupling interval starting at 10 ms greater than the S1-S2 coupling interval at which AV node conduction had failed. If this extrastimulus was conducted successfully from the atrium to the ventricle the protocol was repeated with the S1-S2 coupling interval reduced by 1 ms until AV conduction was lost. The greatest S1-S2 coupling interval that did not conduct after a 1 ms decrement was defined as the AVERP.

**General**

**Histology and immunohistochemistry**

Horses were humanely killed and right atrial biopsies (~ 7 cm) encompassing the AV conduction axis were rapidly excised and frozen. In mice, following humane culling under terminal anaesthesia, right atrial preparations encompassing the AV node were rapidly dissected and frozen. To map the location of the AV node, 5 x 30 μm serial cryosections (in the sagittal plane) per mm of the whole preparation were obtained from horse preparations and 5 x10 μm serial cryosections (in the sagittal plane) per 200 μm of the whole preparation were obtained from mouse preparations. Cryosections were first subjected to Masson’s trichrome staining for morphological identification of regions of interest within the AV conduction. Trichrome Stain (Masson) Light Green Stain Kit (TCS Biosciences, HS773LG) was used following manufacturers instructions and sections were imaged with light microscopy on a Leica LMD6000 using Axiovision (Carl Zeiss Microscopy).

Once the component structures of the AV conduction axis were identified, corresponding cryosections were immunolabelled. In brief, serial sections were fixed in 10% neutral buffered formalin for 30 min and then washed in phosphate buffered saline (PBS) (Sigma-Aldrich, Cat. No. P3813, Lot No. SLBF4171V) 3 times (10 min each). Following this, sections were permeabilised with 0.1% Triton X-100 (Sigma-Aldrich, Cat. No. X-100) for 30 min and washed in PBS again before being blocked with 1% bovine serum albumin (BSA) (Sigma-Aldrich) for 1 h. Sections were incubated with a primary antibody diluted in 1% BSA (HCN4, Alomone Labs, Cat No. APC-052; Cav1.2, Alomone Labs, Cat No. ACC-003) at 4°C for 12 h. Sections were washed in PBS once more before being incubated in an appropriate fluorescein isothiocyanate (ThermoFisher A11008) or Cy3-conjugated IgG secondary antibody (Sigma-Aldrich, AP187C) diluted in 1% BSA at room temperature for 90 min. Antibody concentrations were optimised beforehand. After a final 3 washes in PBS, sections were mounted in VECTASHIELD Antifade Mounting Medium (Novus, Cat. No. H-1000). For each batch of slides, a set of slides were processed identically for optimisation of confocal settings and without primary antibody as a negative control for autofluorescence/background correction.

**Confocal imaging of immunofluorescence and data analysis**

In the first set of experiments,analysis was based on previously described methods.4 Briefly, images were acquired on a Zeiss LCM PASCAL 5 confocal microscope equipped with x10/1.00 Plan Apochromatic objective, x20/1.00 Plan Apochromatic objective and x63/1.00 Plan Apochromatic oil immersion objective. Appropriate lasers (Argon, Cy3 or mercury lamp) and filters (Rhodamine or FITC) were used to excite the fluorophore-conjugated secondary antibodies and images were captured using an Axiocam HRc camera. The confocal parameters were kept identical throughout each experiment for quantification purposes. Three high power images were taken per section. Immunofluorescence was quantified using Volocity cellular imaging software (Quorum Technologies). In all mouse preparations, the anti-Cav1.2 antibody produced aggregates of non-specific high pixel intensity labelling artefact. Fluorescence intensity measurements from these structures were excluded from the analysis based on the ‘exclude-by-intensity’ feature, which successfully identified all aggregates. The sum pixel intensity (in arbitrary units) for the HCN4/Cav1.2-stained area was divided by the area to give a measurement of intensity per unit area. For immunohistochemistry in the horse, fluorescent intensity was quantified on Z-stacks with a total Z-distance of ~40 µm.

**Immunofluorescence imaging in additional horses**

Three sections from each horse AV node were prepared for immunohistochemical staining and imaging. One extra preparation was created for negative control. Images were acquired using a Zeiss AxioImager Z1 widefield microscope coupled to a HXP-120 light source and equipped with an AxioCam 506 mono – 6 Mpx camera, using a Plan Apo 20x/0.5 NA objective with a 570-640 emission filter. To cover the entire nodal area 156 tiles making up ~5x7 mm of sampled field were stitched together. Microscopic settings were kept identical for all samples. Scanned images were analysed using ZEN 2.3 Blue edition (Zeiss, 2011) in combination with the extension software ZEN Intellesis (Zeiss; 2017) - a machine learning-based, pixel classifier tool that was used for image segmentation. The model enabled extraction of 128 pixel-features, allowing for color- and shape-based segmentation of three distinctly defined classes: “Nodal tissue”, “Background” and “Collagen”. The training of the models was performed on sections labelled with both primary antibodies against HCN4 and Cav1.2, generating a model for each. HCN4 positive regions were analysed by drawing around this area using the interactive analysis tool in ZEN 2.3 Blue edition (Zeiss). For Cav1.2 the area was located based on morphological characters and by projecting the HCN4 positive region from the preceding slide. Intensity mean value of channel Cy3 was extracted from the analysis. Staining and imaging was done in batches of 10 slides (all animals represented in each batch). To incorporate data from the two horse cohorts, statistical testing on data presented in Figure 1N,Q was carried out on the average fluorescent intensity per animal calculated from n=3 AV node sections/horse from 7 sedentary horses and n=3 AV node sections/horse from 7 trained horses. To incorporate data from the two separate cohorts, the trained values have been normalised to corresponding sedentary values set at 100%.

**Western blot**

Western blotting was carried out on AV node biopsies (made up of the HCN4-positive compact node) from horses and mice as well as ES-derived nodal cells following overexpression with miR-211-5p and miR-432. Western blotting was carried out as described previously.12 Primary antibodies were the same as described for immunolabelling with the exception of Connexin 45 (Alomone Labs, Cat no. ACC-205). HRP-linked anti-rabbit IgG (Cell Signalling 7074S) was used as a secondary antibody. Protein abundance was normalised to total protein on blots.

**Laser-capture microdissection**

Mouse AV node preparations were dissected, frozen and cryosectioned as given above. 20 μm sections 200 μm apart were subjected to immunolabelling with HCN4 antibody to determine the location and extent of component structures of the AV conduction axis. Based on immunolabelled images, sections were selected and stained with Cresyl Violet to enable visualisation of tissue with light microscopy. In this process, sections were placed in 100% ethanol (3 min), stained with 0.1% Cresyl Violet (4 min), rinsed in tap water, placed in 70% ethanol (2 min), and finally placed in 100% ethanol until laser capture microdissection. Guided by HCN4-positive labelling, the inferior nodal extension, compact node and penetrating bundle were sampled from each preparation using a Leica LMD6000 system and coupled together to represent the entire AV node. Approximately 15 slides per heart were sampled and hearts were not pooled. No obvious variation in the histology or fibrous composition between preparations was noted.

**qPCR**

Gene expression was assessed using Taqman low density array cards for medium throughput profiling of 96 mRNAs or 750 miRs and by single assay SYBR green qPCR.

**Taqman Low Density Array cards**

Total RNA from microdissected samples was isolated using the mirVana RNA Isolation Kit (ThermoFisher Scientific), then quantified using a spectrophotometer, the Nanodrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). Samples were diluted such that 50 ng of total RNA were used in reverse transcription with SuperScript VILO (ThermoFisher Scientific). 96 target transcripts were simultaneously measured from preamplified cDNA using custom-designed TaqMan Low Density Array microfluidic cards (Applied Biosystems, Cat. No. 2549025; format 96A) on an ABI Prism 7900 HT Sequence Detection System (Applied Biosystem Foster City, CA, USA) as described in detail elsewhere.4 Amplification plots were analysed using RQ manager (Life Technologies). Ct values were exported to the RealTime Statminer (Integromics) data analysis package that enabled advanced filtering of outlier genes, geNorm-based selection of the optimal endogenous control genes *Hprt* and *Pgk1* (*Hprt*, *Pgk1, Tbp, Ipo8, Gapdh and 18s* tested), and differential expression testing using the non-parametric Limma test11 followed by Benjamini-Hochberg False Discovery Rate correction.4 Transcript expression levels were calculated using the ΔCt method. **miR profiling**: 65 ng of total RNA from individual AV node samples was reverse-transcribed using TaqMan miR Reverse Transcription Kit (Cat. No. 4366597) and the product (2.5 μl) was preamplified using Megaplex PreAmp Primers Pool A and B (which is a set of pre-defined pools of 380 stem-looped reverse transcription primers) and TaqMan PreAmp Master Mix (Applied Biosystems) in a 25 μl PCR reaction. The preamplification cycling conditions were 95°C for 10 min, 55°C for 2 min and 75°C for 2 min followed by 12 cycles of 95°C for 15 s and 60°C for 4 min. The preamplified cDNA was diluted with 0.1× TE (pH 8.0) to 100 μl and then 10 μl of diluted cDNA was analysed on a TaqMan Array Card run on the ABI Prism 7900 HT Sequence Detection System. qPCR was performed using TaqMan Array Rodent MiR A+B Cards Set v3.0 (ThermoFisher Scientific). Included on each array were three TaqMan miR assay endogenous controls to allow data normalisation and one TaqMan miR assay not related to rodent as a negative control (ath-miR-159a). Results were analysed using RQ manager (Applied Biosystems) and RealTimeStatMiner (Integromics). Data analysis was conducted as described for mRNAs above. A combination of snoRNA135, snoRNA202 and U6 was used for normalisation.

**Single assay qPCR**

cDNA was generated from total RNA extracted using the Qiagen RNeasy Micro Kit from unpooled AV node samples using the miScript II RT kit (Qiagen) and manufacturer’s instructions as described previously.12 Primers were obtained from Qiagen GeneGlobe as follows:

Hcn4: QT01053514; Cav1.2: QT00150752; 18s: QT00291977; Ip08: QT00291977; Hprt: QT00166768; Tbp: QT00198443; miR-211-5p: MS00001897 (mouse and horse); miR-432:MS00017451 (mouse), MS00031850 (horse); Snord61: MS00033705; Snord95 : MS00033726; Rnu62 - MS00033740; Rnu1a1 - YP00203909.

Reference gene combinations for individual experiments were determined using geNorm as given in Online Figure III and are given in accompanying figure legends. Cycling conditions were as follows: mRNA = 95°C for 10 min; 40 cycles of 95°C for 15 s; 60°C for 1 min.

miR = 95°C for 15 min; 40 cycles of 94° C for 15; 55°C for 30 s; 70°C for 30 s. Melt curve analysis was performed at the end of both protocols at the following conditions 95°C 15 s, 60°C for 1 min and 95°C for 15 s. mRNA expression for transcripts was calculated by the ΔCt method.

***Ex vivo* electrophysiology**

AV node function was measured in the isolated AV node using extracellular electrodes. AV node preparations were rapidly dissected while being constantly superfused with oxygenated Tyrode’s solution (100 mM NaCl, 4 mM KCl, 1.2 mM MgSO2, 1.2 mM KH2PO4, 1.2 mM CaCl2, 25 mM NaHCO3 and 11 mM glucose bubbled with 95% O2 and 5% CO2 to give a pH of 7.4.). The preparation was placed in a perfusion bath with oxygenated Tyrode’s solution at 36.5°C. Custom designed bipolar electrodes were used to record signals in the atrium and at the His bundle. The A-H interval was measured as the interval from the peak of an atrial signal to the peak of the following His signal.Wenckebach cycle length and AVERP were assessed using pacing protocols as given above for *in vivo* pacing.

**Myocyte isolation and patch clamp**

Mice were killed by cervical dislocation under general anesthesia with 2% isoflurane in O2 and beating hearts were quickly removed. The AV node region was excised in warmed (35°C) Tyrode’s solution containing (in mM): NaCl, 140; KCl, 5.4; CaCl2, 1.8; MgCl2, 1; Hepes-NaOH, 5; and D-glucose, 5.5 (adjusted to pH 7.4 with NaOH). AV node tissue strips were then transferred into a “low-Ca2+-low-Mg2+” solution containing (in mM): NaCl, 140; KCl, 5.4; MgCl2, 0.5; CaCl2, 0.2; KH2PO4, 1.2; taurine, 50; D-glucose, 5.5; bovine serum albumin (BSA), 1 mg/ml; Hepes-NaOH, 5 (adjusted to pH 6.9 with NaOH). Tissue were digested by adding Liberase TM (0.15 mg/ml, Roche Diagnostics GmbH, Mannheim, Germany), elastase (1.9 U/ml, Worthington, Lakewood, USA). Digestion was carried out for a variable time of 15–18 minutes at 35°C. Tissue strips were then washed and transferred into a modified “Kraftbrühe” (KB) medium containing (in mM): L-glutamic acid, 70; KCl, 20; KOH, 80; (±)D-b-OH-butyric acid, 10; KH2PO4, 10; taurine, 10; BSA, 1 mg/ml; and Hepes-KOH, 10 (adjusted to pH 7.4 with KOH). Single AV node cells were isolated by manual agitation in KB solution at 35°C for 30–50 s. Cellular automaticity was recovered by re-adapting the cells to a physiological extracellular Ca2+ concentration by addition of a solution containing (in mM): NaCl, 10, CaCl2, 1.8 and normal Tyrode solution containing BSA (1 mg/ml). The final storage solution contained (mM): NaCl, 100; KCl, 35; CaCl2,1.3; MgCl2, 0.7; L-glutamic acid, 14; (±)D-b-OH-butyric acid, 2; KH2PO4; 2; taurine, 2; BSA 1 mg/ml (pH=7.4).

**Patch clamp recording from AV node cells**

For electrophysiological recordings, aliquots of the cell suspension were harvested in a 3.5 mm diameter Petri dish and mounted on the stage of an inverted microscope and perfused with normal Tyrode’s solution. The recording temperature was 36°C. The whole-cell variation of the patch-clamp technique was used to record cellular ionic currents and cell automaticity, by employing an Axopatch 200A or 700B (Axon Instruments Inc., Foster USA) patch clamp amplifier. Recording electrodes were fabricated from borosilicate glass, by employing a Narishige PC 830 gravity microelectrode puller (Narishige Group, Tokyo, Japan) or a DMZ-Universal-Electrode-Puller (Zeitz-Instruments Vertriebs GmbH, Martinsried, Germany). Cellular automaticity, *IK,r* and *I*f were recorded under the standard whole-cell configuration during perfusion of standard Tyrode’s solution (for *If* recordings, 2 mM BaCl2 was added to Tyrode’s solution to block *I*K,1) Patch pipettes were filled with an intracellular solution containing (in mM): KCl 130, NaCl 10, EGTA 1, ATP (Na salt) 2, MgCl2 0.5 and HEPES 5 (pH adjusted to 7.2 with KOH). For *I*Ca,*L*, pipette solution contained (in mM): 125 CsOH, 20 tetraethylammonium chloride (TEA-Cl), 1.2 CaCl2, 5 Mg-ATP, 0.1 Li2-GTP, 5 EGTA and 10 HEPES (pH adjusted to 7.2 with aspartate). 30 µM tetrodotoxin (Latoxan, Portes lès Valence, France) was added to external standard Tyrode solution to block *I*Na. Electrodes had a resistance of ~3 MΩ. Seal resistances were in the range of 2–5 GΩ. All electrophysiological data were recorded using pCLAMP 8 (Molecular Devices, Sunnyvale, CA, USA). Data were analysed off-line using Clampﬁt 9.2 (Molecular Devices, Sunnyvale, CA, USA). GraphPad Prism 7.02 (Graphpad Software, La Jolla, USA) and SAS v9.4 (SAS Institute). Data were analysed by initially establishing whether a transformation was required to ensure all assumptions were valid. Examination of residual diagnostics indicated that a log transformation was required. The analysis used a mixed effects linear model, with fixed effects of activity level (sedentary or trained), and voltage (mV) or action potential parameter. The interaction between these two effects was also included. The nested random effects in the model were the ‘animal’ and ‘myocyte within animal ID’. A variance components structure was assumed. Components of variance were estimated from the model of residual variability (unexplained by the model), between myocytes within animal variability and additional variability between animals. The percentage of total variability accounted for by each of the components was also calculated. Note that for action potential measurements there is only one observation for each cell (for each parameter type), the between cells (within animal variability) and the residual variability are inseparable – labelled residual.

The following variability component estimates were obtained:

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Between animals** | | **Between myocytes (within animal)** | | **Residual** | | **Total Variability (σ2)** |
|  | **Log** | **%** | **Log** | **%** | **Log** | **%** |
| ***I*Ca,L (Figure 3B)** | 0.016 | 16.5% | 0.0069 | 7.1% | 0.0742 | 76.4% | 0.0971 |
| ***I*f (Figure 3D)** | 0.065 | 46.4% | 0.0187 | 13.3% | 0.0565 | 40.3% | 0.1402 |
| ***I*K (Figure 4D,E)** | 0 | 0.0% | 0.028 | 57.7% | 0.0205 | 42.3% | 0.0485 |

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Action potential parameters (Figure 5B)** | **Residual** | | **Between animals** | | **Total Variability (σ2)** |
|  | **Log** | **%** | **Log** | **%** |
| **Rate** | 1558 | 100% | 0 | 0.0% | **1558** |
| **SLDD** | 0.0225 | 100.0% | 0 | 0.0% | **0.0225** |
| **SEDD** | 0.0033 | 100.0% | 0 | 0.0% | **0.0033** |

This analysis showed that for *I*K and action potential parameters all the variability was between cells, within animals. We conclude, for these data, that there is no correlation between measurements that are made on the same animal. Therefore, comparisons reported in Figures 4B,D and Figure 5B) are based on a mixed effects linear model where activity status (sedentary or trained), voltage or action potential parameter were designated as fixed effects and myocytes were specified as random effects. For *I*Ca,L and*If* (Figures 3B and 3D) activity status and voltage were fixed effects and animals specified as random effects in a mixed effects linear model. For all datasets, the model included the interaction between factors to determine interdependence. Comparisons were conducted using a 2-sided 5% test, and applying a Sidak multiple comparison adjustment.

**Computer modelling**

To simulate the AV node action potential, the model of the rabbit AV node action potential (N-type) from Inada *et al.*13was used. General equations are:

(1)

*ICa*,*L* +*IK*,*r* +*If*+*Ist* +*Ip* +*INaCa* +*Ib* (2)

where *V* (mV) is the membrane potential, *t* (ms) is the time, *Itotal* (pA) is the total membrane current, and *Cm* (pF) is the membrane capacitance. The total membrane current is the sum of seven ionic currents, which are shown in equation (2). A one-dimensional (1D) model was used to simulate conduction in the AV node. The model includes 100 elements and each element is 100 μm in length. The reaction-diffusion equation used was:

(3)

where *D* is the diffusion coefficient. *D* was set to 0.001 μS mm2. One end of the model (elements 1 to 3) was stimulated at 3 Hz. The conduction velocity was calculated from the times of arrival of the action potential at the 41st and 61st elements to avoid the influence of the boundaries. 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% based on the patch clamp recordings in isolated AV node myocytes as given above.

**Computational prediction of miR targets**

Previously validated target prediction algorithms RNA22, miRDB and Sfold were used to investigate whether differentially expressed miRs identified by TLDA card profiling had predicted binding sites in the 3′UTR of Cav1.2 and HCN4 (**Supplementary Table S5**). 3′UTR sequences were obtained from UCSC Genome Browser.

**Luciferase reporter gene assays**

miR mimics were purchased from Dharmacon. The following 3′UTR luciferase reporter constructs (GeneCopoeia) were used:

p*m*Hcn4-3′UTR-Luc - MmT100218-MT06

p*m*Cav1.2-3′UTR-Luc - MmiT077219a-MT06 (part A), MmiT077219b-MT06 (part B) and MmiT077219c-MT06 (part C)

p*m*NegCtrl-3′UTR-Luc - Negative control 3′UTR plasmid, CmiT000001-MT06

All plasmids contained reporters for both firefly and renilla luciferase. 24 h prior to transfection, 105 H9C2 cells were plated in 48 well plates. Cells were transfected with 0.25 μg wild-type or mutated 3′UTR plasmid and 25 nM of miR mimic, using 1.25 μl of Lipofectamine 2000. Firefly and renilla luciferase were measured at 24 h post-transfection using the Dual-Luciferase Reporter Assay on the GloMax Explorer System (both Promega) according to the manufacturer’s instructions. The luminescence intensity of firefly luciferase was normalised to that of Renilla luciferase.

**Site directed mutagenesis**

Site directed mutagenesis was carried out using the PCR based Quick Change site directed mutagenesis protocol (Stratagene). Primers were designed to make the appropriate mutations as given in Online Figure VII. Reaction mixtures of 50 μl contained 20 ng of template DNA, 125 ng of both forward and reverse mutagenic primers, 1x Pfu PCR buffer, 240 μM dNTPs and 2.5 units *PfuTurbo* DNA polymerase. These reactions were subjected to an initial denaturation at 95 for 30 s, followed by 12 PCR cycles of 95˚C for 30 s, 45˚C for 1 min and 65˚C for 2 min/kb of plasmid. Completed reactions were treated with 10 units *Dpn I* at 37˚C for 2 h to digest the methylated parental DNA after which 5 μl was used to transform chemically competent XL1-Blue strain of *E. coli* (Agilent) by heat shock. Presence of desired mutations was confirmed by Sanger sequencing. A fragment of the plasmid containing the 3′UTR mutations was excised with appropriate restriction endonucleases and subcloned into appropriate vectors for transfection as given above.

|  |  |  |
| --- | --- | --- |
| **SDM Primers** | | |
| **Name** | **Forward primer sequence 5′-3′\*** | **3′UTR site mutated (bp)** |
| HCN4 SDM miR-211-5p and miR-335-3p† | GAGGGAGAGAGGGAGGCTGGGTGGGTGGGGGGAGCAGACAAG† | 219-229 |
| HCN4 SDM miR-432 | TCCCTCTTCTTTTTCTTGGTCGTTTGTTCCTTCAGGTTTAACTGTG | 30-39 |
| Cav1.2 SDM miR-211-5p | CTTCCTCTACAAACCCAGTCAACCGTACAGAGGTGAAAGGAACTC | 4645-4653 |
| Cav1.2 SDM miR-380-5p | GCTTTGAGGAAAATAAGACTGAAGCAATCGCTCAGATTTTGCCTACG | 2319-2326 |
| Cav1.2 SDM miR-432 | CTGGATTTCAAAAGAGCTAGACGAACCCCACGGAAGCTGATGTTAAG | 3985-3993 |
| Cav1.2 SDM miR-215-5p | AGAGGGGTGGGGAGTGAGGAAACGAGAAGCCTTAAACCCCACCACCAC | 4102-4108 |

\*Reverse primers were the reverse complement of the forwards sequence. †This primer was designed to mutate 4 overlapping miR binding sites: 3 sites for miR-211-5p and 1 site for miR-335-3p.

**miR overexpression studies in ES-derived myocytes**

ES-derived myocytes (Shox2, ATCC® CRL­3256™) were maintained in mouse embryonic stem cell basal medium (ATCC) supplemented with 15% fetal bovine serum, 1% penicillin-streptomycin and 0.05 mM β-mercaptoethanol. Cells were passaged using Accutase dissociation reagent (Sigma). For miR overexpression, 1.0x105 cells/well were plated in 24 well plates 24 h prior to transfection with 25 nM miR mimic (Dharmacon). Lipofectamine 2000 (Invitrogen) was used for transfections and cells were incubated for 24 h with Lipofectamine-miR complexes, prior to collection in TRIzol reagent (Invitrogen). RNA was extracted using the PureLink RNA Mini kit (ThermoFisher) according to the manufacturer’s instructions. cDNA was generated using the miScript II RT kit (Qiagen), using the HiFlex buffer option, to allow analysis of miRs and mRNAs in the same cDNA sample. The miScript SYBR green PCR kit was used to measure miR expression. The reaction comprised 1 μl cDNA, 1× miScript universal primer, 1× miScript miR assay, 1× Quantitect SYBR green and DNAse-free water. All samples were run in duplicate. Reaction conditions: denaturation step of 95°C for 15 min followed by 40 cycles of amplification and quantification steps of 94°C for 15 s, 55°C for 30 s and 70°C for 30 s. miR expression was calculated by the ΔCt method with normalisation to expression of *Rnu6-2*, *Snord65* and *Snord91*.

**Statistical analysis**

Animal numbers were estimated *a priori* (while obtaining ethical approval and funding for the studies). This was based on power calculations estimated using StatMate (GraphPad Software, Inc) with 80% power and 95% confidence interval using standard deviation values from our previously published work on cardiac electrophysiological and molecular alterations in swim-trained mice.4,5

Statistical analysis of experimental data was carried out using GraphPad Prism 8 (GraphPad Software, Inc.) and SAS v 9.4 (SAS Institute). Details of individual statistical tests applied are given in the Statistical Analysis table. For all experiments, Residual diagnostics were examined and a Shapiro-Wilk test for normal distribution applied to confirm that the assumptions for the analysis to be valid were applicable.Where data were not normally distributed and/or the number of observations was <7 a non-parametric test (Mann-Whitney test or Kruskal-Wallis test) was used. If data were normally distributed an unpaired Student’s *t* test (two sided) was used to analyse differences between two groups. When the null hypothesis of equal variance was rejected, an unpaired t-test with Welch’s correction was used. To compare multiple normally distributed groups, an ANOVA (one- or two-way) was used. Dunnett’s, Holm-Sidak, Sidak or Tukey’s test were applied for multiple testing correction, automated by the software package used. P<0.05 was regarded as significant. In figures, means±SEM are given in dot plots; asterisks indicate significance. Statistical tests for qPCR data and electrophysiological recordings in isolated AV node myocytes are detailed in the relevant sections above. A limitation of the statistical analysis in this work is that multiple testing correction was only conducted on high-throughput qPCR data (presented in Supplemental tables S2-S4 and in Online Figures I(E) and IV) but not across the entire dataset.

**REFERENCES**

1. Haugaard MM, Pehrson S, Carstensen H, Flethoj M, Hesselkilde EZ, Praestegaard KF, Diness JG, Grunnet M, Jespersen T and Buhl R. Antiarrhythmic and electrophysiologic effects of flecainide on acutely induced atrial fibrillation in healthy horses. *J Vet Intern Med*. 2015;29:339-47.

2. Barold SS and Padeletti L. Mobitz type II second-degree atrioventricular block in athletes: true or false? *Br J Sports Med*. 2011;45:687-90.

3. Barold SS and Hayes DL. Second-degree atrioventricular block: a reappraisal. *Mayo Clin Proc*. 2001;76:44-57.

4. D'Souza A, Bucchi A, Johnsen AB, Logantha SJ, Monfredi O, Yanni J, Prehar S, Hart G, Cartwright E, Wisloff U, Dobryznski H, DiFrancesco D, Morris GM and Boyett MR. Exercise training reduces resting heart rate via downregulation of the funny channel HCN4. *Nat Commun*. 2014;5:3775.

5. D'Souza A, Pearman CM, Wang Y, Nakao S, Logantha S, Cox C, Bennett H, Zhang Y, Johnsen AB, Linscheid N, Poulsen PC, Elliott J, Coulson J, McPhee J, Robertson A, da Costa Martins PA, Kitmitto A, Wisloff U, Cartwright EJ, Monfredi O, Lundby A, Dobrzynski H, Oceandy D, Morris GM and Boyett MR. Targeting miR-423-5p Reverses Exercise Training-Induced HCN4 Channel Remodeling and Sinus Bradycardia. *Circ Res*. 2017;121:1058-1068.

6. Benito B, Gay-Jordi G, Serrano-Mollar A, Guasch E, Shi Y, Tardif JC, Brugada J, Nattel S and Mont L. Cardiac arrhythmogenic remodeling in a rat model of long-term intensive exercise training. *Circulation*. 2011;123:13-22.

7. Duygu B, Juni R, Ottaviani L, Bitsch N, Wit JBM, de Windt LJ and da Costa Martins PA. Comparison of different chemically modified inhibitors of miR-199b *in vivo*. *Biochem Pharmacol*. 2019;159:106-115.

8. Mohamed TM, Abou-Leisa R, Stafford N, Maqsood A, Zi M, Prehar S, Baudoin-Stanley F, Wang X, Neyses L, Cartwright EJ and Oceandy D. The plasma membrane calcium ATPase 4 signalling in cardiac fibroblasts mediates cardiomyocyte hypertrophy. *Nat Commun*. 2016;7:11074.

9. Hagendorff A, Schumacher B, Kirchhoff S, Luderitz B and Willecke K. Conduction disturbances and increased atrial vulnerability in Connexin40-deficient mice analyzed by transesophageal stimulation. *Circulation*. 1999;99:1508-15.

10. Temple IP, Logantha SJ, Absi M, Zhang Y, Pervolaraki E, Yanni J, Atkinson A, Petkova M, Quigley GM, Castro S, Drinkhill M, Schneider H, Monfredi O, Cartwright E, Zi M, Yamanushi TT, Mahadevan VS, Gurney AM, White E, Zhang H, Hart G, Boyett MR and Dobrzynski H. Atrioventricular node dysfunction and ion channel transcriptome in pulmonary hypertension. *Circ Arrhythm Electrophysiol*. 2016;9.

11. Jeanmougin M, de Reynies A, Marisa L, Paccard C, Nuel G and Guedj M. Should we abandon the t-test in the analysis of gene expression microarray data: a comparison of variance modeling strategies. *PloS one*. 2010;5:e12336.

12. Bidaud I, D’Souza A, Forte G, Torre E, Greuet D, Thirard S, Anderson C, Chung You Chong A, Torrente A, Roussel J, Wickman K, Boyett M and Mesirca P. Genetic ablation of G protein-gated inwardly rectifying K+ (GIRK4) channels prevents training-induced sinus bradycardia. *Frontiers in Physiology*. 2021;11:519382.

13. Inada S, Hancox JC, Zhang H and Boyett MR. One-dimensional mathematical model of the atrioventricular node including atrio-nodal, nodal, and nodal-his cells. *Biophys J*. 2009;97:2117-27.

**Supplementary Table S1:** Echocardiography parameters of sedentary mice and mice trained for 5 months and statistical significance of any difference assessed by Student’s *t* test. (Normality was assessed by Shapiro Wilk test and by examination of residual diagnostics on a Normal Quantile plot). LVDd, left ventricular diastolic diameter; BW, body weight; LVDs, left ventricular systolic diameter; dIVS, left ventricle septal wall thickess; dPW, left ventricular posterior wall thickness.

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Sedentary mice (n=15)** | **Trained mice (n=15)** | **P value** |
| LVDd/BW (cm/g) | 0.13 | 0.14 | 2.87 x 10-4 |
| LVDs/BW (cm/kg) | 0.09 | 0.08 | 5 x 10-3 |
| LV Mass/BW | 4.14 | 3.67 | 2.3 x 10-1 |
| Relative wall thickness | 0.37 | 0.39 | 2.2 x 10-1 |
| dIVS/LVDd | 32.58 | 30.97 | 3.9 x 10-1 |
| dPW/LVDd | 19.37 | 18.6 | 4.5 x 10-1 |
| Stroke volume (μl) | 55.2 | 59.88 | 1.6 x 10-1 |
| Ejection fraction (%) | 72.74 | 71.91 | 7.6 x 10-1 |
| Fractional shortening (%) | 34.93 | 35.48 | 7.4 x 10-1 |
| Heart rate (beats/min) | 543.21 | 488.13 | 47.6 x 10-3 |

**Supplementary Table S2:** mRNAs in the mouse AV node that change significantly in response to training. Expression normalised to *Hprt* and *Pgk1* in sedentary and trained mice, statistical significance of the difference, ratio of expression in trained mice:sedentary mice, and n numbers shown.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **mRNA** | **Sedentary mice (mean**±**SEM)** | **Trained mice (mean**±**SEM)** | **FDR adjusted**  **P value** | **Trained/sedentary (%)** | **n** |
| **HCN channels** | | | | |  |
| HCN2 | 2.9 ± 1.4 | 0.32 ± 0.1 | 0.017 | 11% | 7/7 |
| HCN4 | 4.3 ± 0.8 | 0.41 ± 0.1 | 0.002 | 10% | 7/6 |
| **Na+ channels** | | | | |  |
| Nav1.5 | 13.1 ± 6.19 | 2.1 ± 1.3 | 0.018 | 16% | 6/7 |
| SCN1B | 8.7 ± 3.2 | 0.7 ± 0.1 | 0.009 | 8% | 7/7 |
| **Ca2+ channels** | | | | |  |
| Cav1.2 | 45.2 ± 23.4 | 6.1 ± 1.5 | 0.034 | 13% | 6/6 |
| Cav3.1 | 22.8 ± 12.5 | 2.1 ± 0.6 | 0.014 | 9% | 7/6 |
| Cav3.2 | 35.4 ± 14.6 | 3.4 ± 0.2 | 0.016 | 10% | 7/5 |
| Cavα2δ1 | 15.8 ± 4.8 | 3.2 ± 0.6 | 0.017 | 21% | 7/7 |
| Cavα2δ2 | 63.3 ± 38.0 | 3.6 ± 0.6 | 0.018 | 6% | 6/6 |
| **Transient outward K+ channels** | | | | |  |
| KChIP2 | 14.2 ± 7.1 | 0.3 ± 0.1 | 0.047 | 2% | 7/7 |
| Kv4.3 | 2.9 ± 1.2 | 0.6 ± 0.2 | 0.017 | 21% | 7/7 |
| **Delayed rectifier K+ channels** | | | | |  |
| Kv1.2 | 2.0 ± 0.9 | 0.2 ± 0.1 | 0.017 | 9% | 7/7 |
| Kv1.5 | 13.2 ± 5.6 | 1.0 ± 0.3 | 0.018 | 8% | 6/7 |
| Kv2.1 | 12.1 ± 4.8 | 1.0 ± 0.3 | 0.014 | 9% | 7/7 |
| **Inward rectifier K+ channels** | | | | |  |
| Kir2.2 | 23.7 ± 10.3 | 1.7 ± 0.6 | 0.017 | 7% | 7/7 |
| Kir3.4 | 38.9 ± 16.4 | 4.1 ± 0.9 | 0.022 | 11% | 7/7 |
| Kir6.1 | 3.7 ± 1.7 | 0.3 ± 0.1 | 0.017 | 7% | 7/7 |
| SUR2 | 10.4 ± 4.1 | 1.3 ± 0.2 | 0.018 | 12% | 7/6 |
| **Miscellaneous K+ channels** | | | | |  |
| SK1 | 2.8 ± 1.3 | 0.16 ± 0.04 | 0.032 | 6% | 7/7 |
| SK2 | 0.8 ± 0.3 | 0.11 ± 0.03 | 0.022 | 13% | 7/6 |
| SK3 | 0.9 ± 0.3 | 0.11 ± 0.03 | 0.008 | 12% | 7/7 |
| TWIK-1 | 0.3 ± 0.1 | 0.02 ± 0.01 | 0.008 | 7% | 6/7 |
| **Cl- channels** | | | | |  |
| Chloride channel 2 | 1.4 ± 0.6 | 0.12 ± 0.04 | 0.019 | 9% | 6/7 |
| Chloride channel 3 | 20.8 ± 9.5 | 1.0 ± 0.1 | 0.019 | 5% | 6/7 |
| **Gap junction channel** | | | | |  |
| Cx30.2 | 0.5 ± 0.2 | 0.03 ± 0.02 | 0.017 | 5% | 7/7 |
| Cx40 | 3.4 ± 1.2 | 1.1 ± 1.0 | 0.018 | 33% | 7/7 |
| Cx45 | 12.7 ± 5.1 | 1.1 ± 0.3 | 0.013 | 8% | 7/7 |
| **Na+-K+ Pump** | | | | |  |
| Na+-K+ pump α1 subunit | 162.6 ± 82.6 | 22.5 ± 4.1 | 0.031 | 14% | 6/6 |
| Na+-K+ pump α2 subunit | 121.9 ± 53.9 | 4.5 ± 0.6 | 0.016 | 4% | 7/7 |
| Na+-K+ pump α3 subunit | 0.3 ± 0.1 | 0.05 ± 0.01 | 0.019 | 19% | 6/7 |
| **Intracellular Ca2+-handling** | | | | | |
| Phospholamban | 697.5 ± 306.0 | 34.5 ± 7.8 | 0.017 | 5% | 7/7 |
| Calsequestrin 2 | 83.6 ± 37.0 | 8.9 ± 1.5 | 0.044 | 11% | 7/4 |
| RYR2 | 286.9 ± 138.0 | 8.8 ± 0.8 | 0.018 | 3% | 7/5 |
| SLC8A1 | 295.1 ± 135.4 | 12.7 ± 2.9 | 0.018 | 4% | 7/7 |
| Ca2+/calmodulin-dependent protein kinase II | 21.5 ± 7.9 | 3.2 ± 0.2 | 0.007 | 15% | 7/5 |
| **Extracellular matrix components** | | | | |  |
| Collagen type 1 a | 68.1 ± 25.2 | 11.4 ± 3.1 | 0.021 | 17% | 7/6 |
| Fibronectin-1 | 32.0 ± 12.3 | 3.3 ± 1.1 | 0.008 | 10% | 7/7 |
| Interleukin-1β | 0.15 ± 0.08 | 0.007 ± 0.004 | 0.013 | 4% | 7/7 |
| MMP2 | 11.4 ± 4.6 | 1.1 ± 0.3 | 0.018 | 10% | 7/7 |
| MMP9 | 2.3 ± 1.0 | 0.1 ± 0.03 | 0.014 | 4% | 7/7 |
| TGFβ1 | 17.9 ± 10.2 | 0.4 ± 0.1 | 0.018 | 2% | 7/6 |
| TIMP4 | 6.4 ± 2.7 | 0.4 ± 0.1 | 0.018 | 6% | 7/7 |
| Vimentin | 781.3 ± 463.0 | 27.5 ± 8.8 | 0.030 | 4% | 7/7 |
| **Transcription factors** | | | | |  |
| GATA4 | 22.5 ± 7.6 | 4.6 ± 0.9 | 0.018 | 20% | 7/6 |
| IRX3 | 1.7 ± 0.8 | 0.1 ± 0.01 | 0.003 | 6% | 7/7 |
| KLF4 | 6.2 ± 2.1 | 1.5 ± 0.2 | 0.017 | 24% | 7/6 |
| MEF2C | 3.4 ± 1.3 | 0.3 ± 0.1 | 0.014 | 10% | 7/6 |
| NFκβ | 50.6 ± 23.7 | 1.5 ± 0.2 | 0.019 | 3% | 7/7 |
| NKX2-5 | 128.0 ± 58.7 | 3.9 ± 1.1 | 0.030 | 3% | 7/6 |
| REST | 12.7 ± 6.1 | 0.5 ± 0.1 | 0.018 | 4% | 7/7 |
| SP1 | 18.4 ± 7.8 | 2.0 ± 0.3 | 0.042 | 11% | 7/7 |
| TBP | 26.6 ± 13.1 | 0.3 ± 0.1 | 0.026 | 1% | 7/7 |
| TBX18 | 7.1 ± 3.8 | 0.4 ± 0.1 | 0.042 | 6% | 7/7 |
| TBX3 | 8.9 ± 3.5 | 1.2 ± 0.4 | 0.008 | 13% | 7/7 |
| **Miscellaneous** | | | | |  |
| BMP2 | 12.2 ± 7.8 | 1.4 ± 0.4 | 0.017 | 8% | 7/7 |

**Supplementary Table S3:** mRNAs in the mouse AV node that do not change significantly in response to training. Expression normalised to *Hprt* and *Pgk1* in sedentary and trained mice, P value of the difference, and n numbers shown.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **mRNA** | **Sedentary mice (mean**±**SEM)** | **Trained mice (mean**±**SEM)** | **FDR adjusted P value** | **n** |
| **HCN channels** | | | | |
| HCN1 | 12.3 ± 5.1 | 1.8 ± 0.5 | 0.075 | 7/6 |
| **Na+ channels** | | | | |
| Nav1.1 | 0.01 ± 0.009 | 0.002 ± 0.001 | 0.118 | 5/5 |
| **Ca2+ channels** | | | | |
| Cav1.3 | 0.022 ± 0.012 | 0.004 ± 0.001 | 0.923 | 7/5 |
| Cavβ2 | 1.2 ± 0.2 | 0.8 ± 0.1 | 0.157 | 7/7 |
| **Transient outward K+ channels** | | | | |
| Kv1.4 | 0.2 ± 0.03 | 0.1 ± 0.01 | 0.139 | 7/7 |
| Kv4.2 | 7.2 ± 3.6 | 0.5 ± 0.1 | 0.138 | 7/7 |
| KvLQT1 | 0.6 ± 0.1 | 0.5 ± 0.1 | 0.518 | 6/7 |
| ERG-1 | 7.6 ± 1.6 | 3.9 ± 0.8 | 0.083 | 7/7 |
| **Inward rectifier K+ channels** | | | | |
| Kir2.1 | 15.3 ± 7.7 | 0.6 ± 0.2 | 0.077 | 7/7 |
| Kir3.1 | 23.7 ± 12.3 | 3.3 ± 1.3 | 0.056 | 6/6 |
| Kir6.2 | 4.9 ± 2.2 | 1.1 ± 0.3 | 0.054 | 6/7 |
| SUR1 | 6.1 ± 2.4 | 1.5 ± 0.8 | 0.163 | 7/5 |
| **Miscellaneous K+ channels** | | | | |
| TASK1 | 15.8 ± 6.5 | 5.3 ± 1.5 | 0.095 | 7/6 |
| TRPC6 | 0.006 ± 0.002 | 0.003 ± 0.001 | 0.060 | 6/7 |
| **Connexins** | | | | |
| Cx43 | 5.9 ± 3.4 | 0.8 ± 0.1 | 0.611 | 7/7 |
| **Intracellular Ca2+-handling** | | | | |
| SERCA2 | 251.8 ± 60.5 | 84.8 ± 28.0 | 0.101 | 7/3 |
| IP3 receptor 2 | 4.1 ± 1.7 | 0.5 ± 0.05 | 0.090 | 7/5 |
| PMCA1 | 1.6 ± 0.5 | 0.8 ± 0.2 | 0.101 | 7/6 |
| PMCA4 | 7.4 ± 3.4 | 0.8 ± 0.1 | 0.090 | 7/7 |
| Sarcolipin | 331.6 ± 157.3 | 40.8 ± 29.6 | 0.151 | 7/5 |
| **Extracellular matrix components** | | | | |
| Collagen type 3 a | 31.8 ± 18.6 | 3.9 ± 1.0 | 0.054 | 7/6 |
| CTGF | 3.9 ± 0.5 | 3.8 ± 1.0 | 0.704 | 7/6 |
| Elastin | 34.6 ± 14.5 | 4.2 ± 1.1 | 0.051 | 7/7 |
| TIMP1 | 4.4 ± 1.9 | 0.4 ± 0.1 | 0.067 | 7/6 |
| TNF | 0.03 ± 0.01 | 0.01 ± 0.003 | 0.163 | 7/7 |
| **Transcription factors** | | | | |
| KLF15 | 1.6 ± 0.4 | 1.0 ± 0.4 | 0.222 | 7/6 |
| SHOX2 | 0.11 ± 0.05 | 0.06 ± 0.05 | 0.052 | 7/7 |
| **Miscellaneous** | | | | |
| Atrial natriuretic peptide precursor | 1702.1 ± 817.2 | 41.2 ± 14.0 | 0.121 | 6/5 |
| Brain natriuretic peptide precursor | 10.1 ± 3.1 | 9.1 ± 6.9 | 0.188 | 7/7 |

**Supplementary Table S4:** miRs in the mouse AV node that change significantly in response to training. Expression normalised to *snoRNA135*, *snoRNA202* and *U6* in sedentary and trained mice, n numbers, statistical significance of the difference, and fold change (expression in trained mice/expression in sedentary mice) shown.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **miR** | **Sedentary mice (mean**±**SEM)** | **Trained mice (mean**±**SEM)** | **n**  **S/T** | **P** | **Fold change** |
| mmu-miR-432 | 0.005 ± 0.003 | 0.4 ± 0.2 | 6/8 | 0.0339 | 70.42 |
| mmu-miR-452-5p | 0.17 ± 0.1 | 8.8 ± 5.1 | 6/8 | 0.0022 | 51.09 |
| mmu-miR-325-3p | 1.1 ± 0.6 | 53.7 ± 27.3 | 6/8 | 0.0373 | 48.30 |
| mmu-miR-380-5p | 0.02 ± 0.01 | 1.0 ± 0.5 | 6/8 | 0.0288 | 39.47 |
| mmu-miR-374-5p | 1.8 ± 1.1 | 46.6 ± 21.2 | 6/8 | 0.0298 | 25.53 |
| mmu-miR-592-5p | 4.1 ± 1.7 | 61.7 ± 20.4 | 6/8 | 0.0078 | 15.18 |
| mmu-miR-1964-3p | 2.2 ± 1.6 | 31.9 ± 14.9 | 6/8 | 0.0053 | 14.57 |
| mmu-miR-215-5p | 2.1 ± 1.1 | 30.0 ± 8.8 | 6/8 | 0.0038 | 14.02 |
| mmu-miR-335-3p | 1.8 ± 1.5 | 19.5 ± 10.9 | 6/8 | 0.0176 | 11.07 |
| mmu-miR-1958 | 19.5 ± 7.0 | 211.7 ± 91.7 | 6/8 | 0.0335 | 10.83 |
| mmu-miR-298-5p | 1.55 ± 1.4 | 15.5 ± 6.8 | 6/8 | 0.0026 | 10.02 |
| mmu-miR-503-5p | 1.4 ± 1.3 | 12.9 ± 5.8 | 6/7 | 0.0135 | 9.29 |
| mmu-miR-211-5p | 0.2 ± 0.1 | 1.6 ± 1.1 | 6/8 | 0.0110 | 8.17 |
| mmu-miR-208b-3p | 66.5 ± 34.8 | 512.0 ± 220.0 | 6/8 | 0.0205 | 7.70 |
| mmu-miR-1931 | 33.5 ± 14.1 | 250.5 ± 86.4 | 6/7 | 0.0219 | 7.47 |
| mmu-miR-1193-3p | 22.4 ± 19.0 | 163.6 ± 112.9 | 6/7 | 0.0371 | 7.28 |
| mmu-miR-142-3p | 0.003 ± 0.001 | 0.02 ± 0.01 | 6/8 | 0.0386 | 6.48 |
| mmu-miR-16-1-3p | 7.2 ± 4.6 | 46.4 ± 19.7 | 6/8 | 0.0271 | 6.45 |
| mmu-miR-463-5p | 0.0006 ± 0.0001 | 0.004 ± 0.002 | 6/8 | 0.0256 | 6.32 |
| mmu-miR-881-3p | 2.7 ± 2.1 | 17.3 ± 7.3 | 6/7 | 0.0406 | 6.30 |
| mmu-miR-743b-5p | 5.9 ± 3.4 | 36.0 ± 11.5 | 6/8 | 0.0346 | 6.07 |
| mmu-miR-1947-3p | 15.5 ± 15.0 | 93.3 ± 85.3 | 6/8 | 0.0348 | 6.00 |
| mmu-miR-467e | 17.9 ± 11.0 | 101.4 ± 48.6 | 6/8 | 0.0296 | 5.65 |
| mmu-miR-707 | 6.3 ± 3.5 | 28.5 ± 10.4 | 5/6 | 0.0065 | 4.53 |
| mmu-miR-327 | 0.005 ± 0.002 | 0.024 ± 0.008 | 6/8 | 0.0364 | 4.53 |
| mmu-miR-1959 | 0.0008 ± 0.0002 | 0.004 ± 0.001 | 6/8 | 0.0291 | 4.46 |
| mmu-miR-544-3p | 0.003 ± 0.001 | 0.012 ± 0.003 | 6/8 | 0.0054 | 4.24 |
| mmu-miR-540-5p | 36.2 ± 28.5 | 127.7 ± 39.0 | 6/8 | 0.0214 | 3.53 |
| mmu-miR-615-3p | 39.8 ± 39.8 | 127.3 ± 43.1 | 4/8 | 0.0209 | 3.19 |
| mmu-miR-1897-3p | 0.0002 ± 0.0001 | 0.0006 ± 0.0001 | 6/8 | 0.0312 | 3.008 |
| mmu-miR-105 | 83.8 ± 66.9 | 140.2 ± 50.1 | 5/8 | 0.0377 | 1.67 |
| mmu-miR-693-5p | 121.1 ± 61.8 | 59.2 ± 43.1 | 4/8 | 0.0193 | 0.48 |
| mmu-miR-541-5p | 0.03 ± 0.01 | 0.012 ± 0.006 | 6/8 | 0.0462 | 0.358 |