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An age-associated decline in the role of the sarcoplasmic reticulum & associated calcium-handling proteins sets the pace for sinoatrial node function.

Running title: Ageing causes calcium handling dysfunction in the SAN

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Key Words:

Ageing; arrhythmia; calcium; sarcoplasmic reticulum; sinoatrial node.

Abbreviations:

βAR - β adrenergic response, **BPM**, Beats per Minute, **Ca^{2+}**, Calcium ions, **$Ca_v1.2$**, L-type calcium channel, **CICR**, Calcium Induced Calcium Release, **CPA**, Cyclopiazonic acid, **CT**, Crista terminalis, **LA** - Left atria, **NCX**, Sodium Calcium Exchanger, **PMCA** – Plasma Membrane Calcium ATPase, **RA -** right atria, **RYR** – Ryanodine Receptor, **SAN** – Sinoatrial node, **SERCA** – Sarcoendoplasmic reticulum Ca^{2+} ATPase, **SR** – Sarcoendoplasmic reticulum

Abstract

With advancing age, the intrinsic function of the sinoatrial node (SAN) declines, due to structural changes and changes in electrical regulation within the constitutive cells of the nodal tissue. This study examined changes to proteins involved in regulating calcium flux balance in the atria and SAN of male rats used as a model of ageing throughout their lifespan at 6, 12 and 24 months of age. Using immunohistochemistry and western blot, we determined a significant age-dependent decline in the levels of key calcium regulatory proteins within the SAN: $Ca_v1.2$, PMCA4, RYR2, SERCA2a, and phospholamban (n=5; ANOVA; $p<0.05$). In contrast, levels of NCX protein were significantly elevated by 57.3% ($p = 0.009$) in the oldest group indicating a potential pronounced change in calcium balance; a difference functionally observed by a steeper dose-response curve to the inhibitory effects of nifedipine. Intrinsic pacemaker beating rate was significantly reduced by 68 beats per minute in the oldest group compared with the youngest, (n = 6; ANOVA $p = 0.022$). Negating sarcoplasmic reticulum calcium cycling and the 'calcium clock' using cyclopiazonic acid reduced the intrinsic pacemaker activity of the SAN in young animals to that observed in the oldest group. Under these conditions, spontaneous activity and response of the SAN to isoprenaline became matched across all age groups. Restoring sarcoplasmic reticulum function to the SAN in the elderly may offer a route to combatting age-related suppression of function, but care should be taken in the use of calcium channel antagonists to avoid precipitating sick-sinus syndrome.

Key Points:

- This study adds understanding and characterisation of the age-dependent progressive reduction in sinoatrial node (SAN) function.
- We compared atria and the sinoatrial node across the lifespan of a Han-Wistar rat model of ageing with animals studied at 6, 12 and 24 months of age.
- Sinoatrial node tissue from old rats showed significantly reduced expression of key cellular calcium regulatory proteins compared with nodal tissue from young rats.
- The sinoatrial node from old rats exhibited a slowed intrinsic beating rate and an altered response to drugs that modulate cellular calcium handling.
- Eliminating sarcoplasmic reticulum calcium handling makes the sinoatrial node of a young animal mimic features of an aged animal.

Abstract Figure legend

Stable, responsive pacemaking in the sinoatrial node is driven by the activity of the funny current (membrane clock), interplay of calcium cycling and release from the sarcoendoplasmic reticulum with depolarising sodium-calcium exchange current (calcium clock). With increasing age key proteins associated with calcium cycling are reduced limiting the role of the calcium clock and it's ability to deal with situations that exacerbate calcium buffering requirements such as adrenergic stimulation and modulation of calcium channels. The net effect is an increasing risk of sinoatrial node instability and dysfunction with ageing.

Introduction

The sinoatrial node (SAN) is a spontaneously active, heterogeneous region of tissue located within the intercaval region of the right atrium of the heart serving as the heart's primary pacemaker. The intrinsic automaticity of the SAN and responsiveness to β-adrenergic stimulation declines in an age-correlated manner contributing to a reduction in functional capacity and ability to respond to stress, as well as potentially increasing risk of arrhythmias such as atrial fibrillation in later life (Turner *et al.*, 1999; Fleg *et al.*, 2005; Fang *et al.*, 2007). Within 20 years it is estimated over a third of the UK population will be ≥ 65 years of age with > 10 % diagnosed with atrial arrhythmias, (ONS, 2025) resulting in spiralling NHS costs (Burdett & Lip, 2022). The onset of such arrhythmias is particularly pronounced in males where the impact seems to become evident at an earlier age so this group has been the initial focus of our study (Keller & Howlett, 2016). The aim of this study was to add to our knowledge of the age-dependent progressive deterioration in SAN function in order to identify mechanisms predisposing to rising dysfunction and risk of arrhythmias, with potential routes to preventative and direct treatment.

An unstable membrane voltage drives pacemaking within SAN myocytes. Hyperpolarisation-activated ion channels pass depolarising I_f current contributing to spontaneous depolarisation of SAN myocytes, supplemented in the late phase of diastole by spontaneous releases of calcium from the sarcoplasmic reticulum (SR) (Bers & Lakatta, 2023). These calcium releases drive a small amount of sodium calcium exchange current (I_{NCX}) enhancing the rate of depolarisation to the membrane threshold for triggering a full action potential. This latter component of depolarisation is referred to as the 'calcium clock' and compliments the overall 'membrane 'clock' in ensuring a robust capability for the heart rate, as driven by the SAN, to respond dynamically to stress. As such it is recognised that the balance of calcium ion flux both across the cell membrane and within the sarcoplasmic reticulum of the cells of the SAN is a key component controlling SAN pacemaker function (Yue *et al.*, 2020; Bers & Lakatta, 2023).

Adrenergic stimulation predominantly increases the heart rate directly or indirectly by causing elevations in cAMP. Numerous components key to SAN function are sensitive to cAMP and its actions in either directly changing ion-channel kinetics (e.g. by enhancing activation of If) or subsequently by its impact on calcium regulation (e.g. by increasing calcium loading of the SR by alterations to phospholamban and the calcium current as well as the overall action potential)(Tsutsui *et al.*, 2018; Bers & Lakatta, 2023). We have previously shown that pacemaker cells from the Guinea Pig SAN show an age-associated

decline in $Ca_v1.2$ protein expression, (Jones *et al.*, 2007) which is key to potentially limiting depolarisation of the cells but also can reduce calcium-loading of the SR and so modulate the calcium clock. We have now sought to track age-associated changes in other key calcium handling proteins that impact the SAN 'calcium clock' as we aim to further understand age-related changes in the basal function of the SAN and response to adrenergic stimulation. Our study hypothesis was that a reduced basal beating rate and dynamic response to adrenergic stimulation in the SAN of aged individuals is due to a reduced capacity for dynamic response of the 'calcium clock' limiting its ability to facilitate cardiac acceleration.

Materials and Methods

Ethical approval

In accordance with our Institution's animal welfare committee guidelines animals had free access to water and food, appropriate housing, and were monitored to ensure they were healthy and displayed normal daily activities. Animals were euthanised by anaesthetic overdose (Euthatal - Sodium Pentobarbital i.p.), and death confirmed. All animal experimental procedures were performed in accordance with Home Office UK guidelines (United Kingdom Animals (Scientific Procedures) Act, 1986) with ethical review and approval from the local ethics committee (reference number, UoH U002).

Sinoatrial node isolated from rats.

Male Han-Wistar rats (Charles River, UK) fed standard chow were randomised to differing age categories and raised to their appropriate age in caged social groups. Our rat model of ageing examined rats across their lifespan at the ages of 6 (young), 12 and 24 (old) months. Animals were euthanised at the appropriate age and the heart rapidly excised and immersed in oxygenated Tyrode solution with 95 % O_2/5 % CO_2, pH 7.4, 37°C. For experiments involving mapping the activity of the SAN the right atrium dissected from the heart and opened to expose the intercaval region and nodal tissue (Jones *et al.*, 2004). Tissue architecture defined the SA node tissue as detailed in figure 1.

Figure 1. Endocardial surface of the right atria

Flanked by the right atrial appendage muscle and septum tissue, the dashed lines indicate location of the crista terminalis (CT) and sinoatrial (SA) node tissue regions, between the upper the superior vena cavae (SVC) and lower inferior vena cavae (IVC). Scale bar, 5mm

Extracellular electrode recordings

We used our published approach for recording sites of initiation and spread of activity across the SAN (Jones *et al.*, 2004). Extracellular bipolar electrodes were positioned on the SAN leading pacemaker site (earliest point of activation) and pectinate muscle of the neighbouring right atrial tissue (Fig. 1) to record nodal activation (Neurolog bridge amplifier (Digitimer, UK); Digidata Axon 1440A with Clampex 10 (Molecular devices, UK)) and determine the intrinsic heart rate (IHR). Movement of one of the electrodes in 1 mm steps across the x and y ranges of the tissue permitted a full map of activation timing to be produced across the tissue. Oxygenated Tyrode solution maintained the tissue at a flow rate of 40ml/min with temperature monitored and controlled at 37 °C. The kinetics of pacemaking

are sensitive to temperature so it is important for close regulation (+/- 0.5 degrees) to ensure stable pacemaking in the normal physiological range. Pharmacological agents, isoprenaline hydrochloride, nifedipine and cyclopiazonic acid (CPA) (Sigma-Aldrich) were made to 1 mM/L stock solutions before dilution to the required concentration in Tyrode solution. For each drug dose the preparation was perfused for a minimum of 15 minutes to allow equilibration and to establish the steady-state IHR prior to recording. All chemicals were purchased from Merck Life Science UK Limited.

Analysis of protein expression

Using our previously published method (Jones *et al.*, 2004), tissue was frozen, and a lysate produced for western blot (WB). Proteins were separated using SDS-PAGE, transferred to PVDF and incubated overnight at 4°C with the primary antibodies at 1:1000 dilution followed for 1 hour by incubation with the appropriate secondary antibody conjugated to HRP (Dako, UK) (Table 1). A Bio-Rad Molecular Imager Chemi Doc XRS+ was used to image band densities and associated values determined using ImageJ 1.54g (NIH, USA). Density values were normalised to an appropriate housekeeper protein to control for equal protein loading per sample.

Table 1. Primary antibodies.

Protein	Cat. No.	Company	Country	Primary host	Secondary antibody
$Ca_v1.2$	ACC-003	Alomone Labs	Israel	Rabbit	Swine Anti-rabbit
$Ca_v1.3$	ACC-005	Alomone Labs	Israel	Rabbit	Swine Anti-rabbit
$Ca_v3.1$	ACC-021	Alomone Labs	Israel	Rabbit	Swine Anti-rabbit
SERCA2	MA3-910	Thermo Scientific	UK	Mouse	Rabbit anti-mouse
PMCA4	MA1-914	Thermo Scientific	UK	Mouse	Rabbit anti-mouse
RYR2	MA3-916	Thermo Scientific	UK	Mouse	Rabbit anti-mouse
NCX1	MA3-926	Thermo Scientific	UK	Mouse	Rabbit anti-mouse
PLB	A010	Badrilla	UK	Mouse	Rabbit anti-mouse
Desmin	M 0760	Dako	Denmark	Mouse	Rabbit anti-mouse

As previously described for immunohistochemistry (IHC) (Jones *et al.*, 2004), 10µm frozen tissue slices were incubated overnight at 4ºC with the primary antibodies (1:250 dilution, except for that for PLB which was used at 1:100). Tissue was incubated for 2 hours with a secondary antibody conjugated to Alexa Fluor™ 488 antibody and wheat germ agglutinin conjugated to rhodamine (2BScientific, UK). Labelled proteins were imaged using a plan apochromat 40x/1.3 oil iris M27 lens on a LSM 710 confocal with images captured using Zen software 3.12 (Zeiss Microscopy, Ltd.; U.K). Images were processed within the Zen software to allow separation of the fluorescent channels corresponding to the two labels before performing densitometric assessment of fluorescence intensity for regions. To control for

background signal an unlabelled parallel section of tissue from the sample was also measured using the same approach and the result subtracted. For the protein of interest IHC labelling was collected on the same settings across each age group. Levels of fluorescent labelling are shown in some cases normalised relative to the mean value as determined in tissue from animals at 6 months of age.

Quantitative polymerase chain reaction (QPCR)

We used the RNeasy Fibrous Tissue Mini Kit (Qiagen) to extract mRNA from samples of the rat right atria. Extracted mRNA concentration was assessed by absorbance (NanoDrop 1000) prior to performing qPCR. Reverse transcriptase (Invitrogen U.K) reverse transcribed RNA to complementary DNA (cDNA). Samples of cDNA were stored at 2-4°C for a maximum of 3 days. We detected the cDNA within each sample using a sequence-specific DNA probe. Primers were Desmin (rat NM_022531.1) and GAPDH (rat NM_017008.3) from Invitrogen UK. Transcript primers were optimised to ensure a cycle threshold (CT) value of 29 or less, confirming a strong positive reaction indicative of abundant target nucleic acid. An Applied Biosystems StepOne Plus qPCR machine was used with StepOne software:QPCR to quantify expression in each sample.

Statistics:

Data are presented as mean ± SD. Statistical significance was evaluated by Student's t-test, One-way ANOVA or two-way repeated-measures (RM) ANOVA with Holm-Sidak post-hoc comparisons as appropriate. Alternatively, a Kruskal-Wallis comparison was used where required and indicated. $P<0.05$ was taken as statistically significant. Curve fits and statistical analysis were performed in GraphPad Prism 10 or Excel as appropriate. Unless otherwise stated, n = number of animals. Data, where appropriate, is shown relative to that obtained from samples from the youngest age group at 6 months of age. For protein analysis it is taken that the 6 months age group possessed 100 % of the specific protein of interest to enable calculation of % change relative to that age point.

Results

Age-associated changes in morphological characteristics

We compared tissue obtained from healthy male rats across three age groups: 6, 12 and 24 months of age (Table 2). Data showed rat body weight gradually increased in an age-correlated manner (r^2 = 0.3966; p = 0.0003 by linear regression). A comparison of rats aged 6 and 24 months revealed a significant increase in body weight from 517 ± 35 g to 661 ± 125 g (n = 11; p = 0.000772) as body weight significantly increased between the ages of 6 and 12 months (p = 0.0416), 12 and 24 months (p = 0.0120). In contrast heart weight did not change significantly from 1.55 ± 0.20 g across the age groups of 6, 12 and 24 months (Table 2; ANOVA p = 0.375).

Rats of 6 months and 12 months of age exhibited no significant difference in their heart-to-body weight ratio (ratio of 6-month rat 2.94 ± 0.33 x10^{-3}; n = 6; t-test p = 0.244). Whereas rats aged 24 months revealed a significant reduction in their heart-to-body weight ratio (2.5 ± 0.27 x10^{-3}) when compared with the other age groups of 6 months and 12 months (Table 2; n = 11; 24 vs 6 months ANOVA; p = 0.000481; 24 vs 12 months p = 0.000113).

Table 2. Morphological characteristics of the ageing rat model.

	6 months	**12 months**	**24 months**
Body weight (g)	517 ± 35	556 ± 40	661 ± 125 *****
Heart weight (g)	1.55 ± 0.20	1.64 ± 0.07	1.63 ± 0.18
Heart: Body (x10^{-3})	2.94 ± 0.33	2.96 ± 0.10	2.5 ± 0.27 **‣¥**
Number in group (n)	11	6	11

***** p=0.000772, 24 months compared with 6 months of age.

‣ p= 0.000481, 24 months compared with 6 months of age.

¥ p= 0.000113, 24 months compared with 12 months of age.

Conforming a suitable housekeeper reference for our ageing studies

We determined mRNA expression of the muscle-specific protein desmin and metabolic enzyme Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for variance with increasing age (Table 3) using our qPCR protocol. Maximum cycle threshold (C_T) differences in the right atrial tissue for desmin RNA was 0.3 C_T with no significant changes over the examined age range (n = 5; ANOVA p = 0.314), whereas GAPDH significantly reduced by 1.0 C_T with ageing (n = 5; ANOVA, 6 vs 24 months p = 0.024; 12 vs 24 months p = 0.021). We also examined desmin protein levels in paired right atrial (RA) and left atrial (LA) tissue obtained from rats at 6, 12 and 24 months of age: There was no significant change in desmin levels (n = 5; ANOVA, p = 0.691), thus desmin was used as the reference housekeeper protein for western blotting in this study.

Table 3. Desmin and GAPDH RNA values in right atrial tissue from rats of increasing ages.

Animal age (months)	RNA C_T values (Mean ± SD)	
	Desmin	GAPDH
6	23.6 ± 0.45	21.8 ± 0.55
12	23.9 ± 0.35	21.8 ± 0.49
24	23.6 ± 0.47	20.8 ± 0.35

Table 4. Desmin protein density

Animal age (months)	Tissue type	Desmin protein (Density/pixel)
6	Left atria	3.15 ± 0.25
12	Left atria	3.12 ± 0.19
24	Left atria	3.88 ± 0.58
6	Right atria	3.53 ± 0.71
12	Right atria	2.83 ± 0.40
24	Right atria	3.00 ± 0.59

Age-associated changes in calcium channels in the rat right atria

Immunohistochemistry (IHC) as performed on RA tissue from rats aged 6 and 24 months, showed age-associated changes in the proteins $Ca_v1.2$, $Ca_v1.3$ and $Ca_v3.1$ (Fig. 2). Normalised to the young, the old rat $Ca_v1.2$ protein levels significantly declined to 50.2 ± 4.72 %, whereas $Ca_v1.3$ and $Ca_v3.1$ levels increased by 81.6 ± 10.14 % and 43 ± 23.42 % respectively (mean ± S.D.; ANOVA; p = 0.0001).

FIGURE 2 BELONGS HERE

Figure 2. Changes in IHC calcium channel label within RA at 24 months of age.
A, optical slices of RA tissue from rats at 6 and 24 months of age with fluorescent labelling of $Ca_v1.2$, $Ca_v1.3$ and $Ca_v3.1$. Confocal images, scale bar = 20 µm. **B**, data shown as mean (± S.D.) difference per calcium channel, overlaid with raw data points. Where n = rats per group, $Ca_v1.2$ (n = 10), $Ca_v1.3$ (n = 5) and $Ca_v3.1$ (n = 9) with statistically significant changes in each case relative to that seen in tissue from young animals (ANOVA; p = 0.0001).

Age-associated changes in Cav1.2 protein levels across the rat atria

RA and LA tissue from rats aged 6, 12 and 24 months were analysed by western blot (Fig. 3). $Ca_v1.2$ protein levels in the RA showed a significant drop with increasing age to 53.6 ± 14.1% of that in the young (n = 5; ANOVA, p = 0.011), but in the LA no significant change in expression was seen (p = 0.051).

FIGURE 3 BELONGS HERE

Figure 3. Age-associated changes of $Ca_v1.2$ protein across the atria.
A, Illustrative blot of $Ca_v1.2$ protein levels per age group for both the RA and LA. **B**, data shown as mean ± S.D. per age group, overlaid with raw data points. (n = 5 animals at each age; ANOVA, * p = 0.011 for 24 vs. 6 months).

Age-dependent decline in $Ca_v1.2$ ion channel expression in the sinoatrial node

Western blot analysis of $Ca_v1.2$ channel protein expression within the SAN of rats at 12 months of age showed no significant differences to that found in the node of those aged 6 months (100 ± 24.4 %; Fig. 4A; ANOVA p = 0.207). At 24 months though $Ca_v1.2$ expression levels had significantly declined to 59.6 ± 18.6 % of that observed in the 6 months age group (Fig. 4A; ANOVA, 12 vs 24 months p = 0.004, 6 vs 24 months p=0.0315). We observed a striated pattern by IHC $Ca_v1.2$ protein labelling within SAN tissue in all age groups, however the amount of labelled protein observed significantly declined from 6 months (100 ± 2.2 %) to 55.6 ± 5.8 % at 24 months of age (Fig. 4B; $p = 2.53 \times 10^{-7}$). Data therefore consistently shows a substantial age-dependent decline of $Ca_v1.2$ protein expression within the SAN region in hearts once animals reach 24 months of age compared with that identified within the young age group (6 months).

Modulation of $Ca_v1.2$ function and activity of the sinoatrial node

The intrinsic heart rate of the isolated SAN was assessed and impact of blockade of $Ca_v1.2$ channels determined by application of the selective channel blocker nifedipine across a dose range of 0.1µm - 30µm. Dose-response relationships for nifedipine's actions in each age group were established using a Hill curve to fit the intrinsic heart rate data and the nifedipine dose, plotted using a standard $\log_{10}$ scale for each concentration trialled (Fig. 4C). The SAN from the youngest age group of 6 months showed a dose-dependent response to nifedipine with Hill slope coefficient of 0.94; the maximum dose of 30µM nifedipine caused all spontaneous activity to cease. The SAN from the oldest age group of 24 months had a reduced response to 0.1µM nifedipine, 0.3µM caused a dramatic decline in beating rate with complete cessation at 1µM nifedipine. The dose-response relationship in the nodes from animals at 24 months of age had an average Hill slope coefficient of 4.2. The IC50 for nifedipine in the older age group was not significantly different to that seen in the young (IC50; 1.14 µM at 6 months vs. 1.23 µM at 24 months). Therefore, although the inhibitory binding of nifedipine for $Ca_v1.2$ is comparable for each age group, the impact on beating rate within the old age group has an approximate four-fold steeper relationship than that seen at a young age.

FIGURE 4 BELONGS HERE

Figure 4. Age-associated changes of $Ca_v1.2$ protein and activity within the SAN.

A, Illustrative blot of $Ca_v1.2$ protein levels in the RA at 6, 12 and 24 months. Data shown as mean ± S.D. with raw data points. (n = 5 per group; 12 vs 24 months p = 0.004, 6 vs 24 months p =

335 0.0315 by ANOVA). **B**, optical slices from SAN of rats aged 6 and 24 months with $Ca_v1.2$ protein
336 labelled in green and membranes labelled with wheat germ agglutinin (red). Scale bar = 50 µm.
337 Quantitated labelling density per group with raw data is shown below. (mean ± S.D.; n = 5 animals
338 per age group; 6 vs 24 months $p = 2.53 \times 10^{-7}$ ANOVA); **C**, Hill equation fitted to intrinsic heart rate
339 from rats aged 6 months and 24 months with incrementing concentrations of nifedipine (shown as
340 log_{10}). (n = 6 per group; IC50 = 1.14 µM at 6 months vs. 1.23 µM at 24 months).
341
342

Age-dependent changes of calcium handling protein within the sinoatrial node

Faced with a reduced capacity for calcium influx it is reasonable to expect extrusion pathways may be modulated to maintain calcium balance in nodal tissue from elderly animals. Intracellular balance of Ca^{2+} is maintained by extrusion via sodium-calcium exchanger (NCX1) and plasma membrane calcium ATPase 4 (PMCA4) with sarcoendoplasmic reticulum Ca^{2+} ATPase2a (SERCA2a) maintaining internal calcium buffering by pumping calcium back into the SR store although this is modulated by the inhibitory action of phospholamban (PLB).

Analysed by western blotting NCX1 protein levels within the SAN did not alter significantly between the ages 6 to 12 months, but in rats aged 24 months NCX1 levels were significantly increased to 157 ± 21.5 % of that found in tissue from the 6-month group with levels also being elevated significantly relative to that seen at 12 months too (Fig. 5B; 12 vs 24 months $p = 0.024$, 6 vs 24 months $p = 0.00856$; ANOVA). Analysis of immunolabelling of NCX1 protein within the SAN region identified a similar significant rise with age to 142 ± 15.6 % at 24 months compared with 100 ± 12.8 % at 6 months of age (Fig. 6; $p = 0.0008$). Using western blot we identified PCMA4 calcium pump protein levels within the SAN increased significantly from 6 to 12 months to 122 ± 17.4% but exhibited a significant decline to 78 ± 3.0 % of the amount at 6 months in rats aged 24 months (Fig. 5C; 6 vs 12 months $p = 0.029$; 12 vs 24 months $p = 0.002$; 6 vs 24 months $p = 0.010$). IHC labelling of PCMA4 within the SAN region also showed a similar significant decline with age to 76.9 ± 16.3 % at 24 months compared with 100 ± 11.1 % at 6 months of age (Fig. 6; $p = 0.0157$).

Both ryanodine receptor 2 (RYR2) and SERCA2a also showed significant changes in expression with age. Western blot showed the RYR2 protein levels rose significantly to 135 ± 18.8 % within the SAN from rats aged 12 months compared with tissue from those aged 6 months, but then in rats aged 24 months levels significantly declined to 68 ± 23.2 % of the level in tissue from rats at 6 months of age (Fig. 5D; 6 vs 12 months $p = 0.014$, 12 vs 24 months $p = 0.0005$; 6 vs 24 months $p = 0.029$). IHC labelling of RYR2 protein within the SAN region also showed a significant decrease to 72.6 ± 13.4 % at 24 months compared with 100 ± 13.7 % at 6 months of age ($p = 0.006$). SERCA2a levels significantly increased to 144 ± 39.4 % within the SAN from rats aged 12 months compared with those aged 6 months but then declined significantly to 64 ± 14.7 % in tissue from rats at 24 months of age (Fig. 5E; 6 vs 12 months $p = 0.035$, 12 vs 24 months $p = 0.003$; 6 vs 24 months $p = 0.002$). IHC labelling of SERCA2a within the SAN region also showed a significant decrease with age to 71.7 ± 15.3 % at 24 months compared with 100 ± 14.3 % at 6 months of age (Fig.6; $p = 0.008$).

FIGURE 5 BELONGS HERE

Figure 5. Effect of ageing on levels of calcium handling proteins expressed in the sinoatrial node. A, illustrative western blot of nodal tissue examined for each protein of interest per age group with the desmin housekeeper. **B-D**, Mean ± S.D. per age group (n = 5 animals per group) overlaid with raw data points for nodal expression levels of calcium handling proteins; **NCX1** (5B; ANOVA 12 vs 24 months * p = 0.024, 6 vs 24 months $^{\#}$p = 0.00856), **PMCA4** (5C; ANOVA 6 vs 12 months *p = 0.029; 12 vs 24 months $^{\blacktriangle}$p = 0.002; 6 vs 24 months $^{\#}$p = 0.010), **RYR2** (5C; ANOVA 6 vs 12 months *p = 0.014, 12 vs 24 months $^{\blacktriangle}$p = 0.0005; 6 vs 24 months $^{\#}$p = 0.029), and **SERCA2A** (5E; 6 vs 12 months *p = 0.035, 12 vs 24 months $^{\blacktriangle}$p = 0.003; 6 vs 24 months $^{\#}$p = 0.002).

FIGURE 6 BELONGS HERE

Figure 6. Age-dependent changes of proteins labelled within the sinoatrial region. **A**, optical slice of each section taken through the sinoatrial node regions from rats aged 6 and 24 months were labelled to show expression of specific proteins of interest (green), and all membranes (using wheat germ agglutinin, red). Confocal images scale bar = 50 μm. **B**, quantified fluorescence from IHC expressed relative to signal density from labelled sections of nodal tissue from rats at 6 months of age (Mean ± S.D.; n=5 animals; Student's t-test, NCX1 * p = 0.0008; PMCA4 $^{\blacktriangle}$p = 0.0157; RYR2 $^{\#}$p = 0.006 , SERCA2A $^{\lozenge}$p = 0.008. and total PLB ♠ p = 0.035.

Age-dependent changes in expression of phospholamban in the sinoatrial node.

PLB is expressed as monomer but can dynamically establish a pentameric assembly which disassembles upon phosphorylation in response to adrenergic stimulation whereupon the monomeric form serves as a more effective regulatory inhibitory protein modulating SERCA pump activity (MacLennan & Kranias, 2003). We quantified monomeric PLB protein levels within the SAN (Fig. 7B) and these did not significantly alter between the ages of 6 and 12 months ($p = 0.38$). Monomeric PLB expression however significantly fell in SAN tissue from rats aged 24 months to 58.6 ± 11.3 %, with this fall being significant when compared with levels in SAN from rats at 6 and 12 months of age ($n = 5$; 6 vs. 24 months, $p = 0.0016$; 12 vs. 24 months, $p = 0.0017$).

By comparison pentameric PLB protein levels rose from 100 ± 20.8 % at 6 months to 119.1 ± 22.3 % at 12 months of age ($p = 0.09$), then in rats aged 24 months significantly declined to 72.4 ± 21.0 % (Figure 7C, 12 vs. 24 months $p = 0.004$; 6 vs. 24 months $p = 0.035$). Total PLB levels did not alter from 100% between the ages 6 to 12 months ($p= 0.47$) but significantly decreased to 68.9 ± 19.3 % in rats aged 24 months (Figure 7D, 12 vs. 24 months $p = 0.0120$; 6 vs. 24 months $p = 0.0123$). This age-dependent decrease between young and old rats was also observed in our total PLB protein immunolabelling of the SAN, as label density significantly fell from 6 months (100 ± 16.5 %) to 78.7 ± 15.7 % at 24 months (Figure 6, 6 vs. 24 months, $p = 0.035$). The ratio of SERCA pump protein to PLB monomer (SERCA : PLB) is considered indicative of activity and was determined as 1.0 ± 0.31 at 6 months and significantly rose at 12 months to 1.5 ± 0.148, indicative of greater tonic SERCA inhibition (Fig. 7E; 6 vs. 12 months $p = 0.0080$). From 12 months onwards the SERCA : PLB ratio declined significantly to 1.2 ± 0.17 at 24 months (12 vs. 24 months $p = 0.0125$). The SERCA : PLB ratio observed in tissue from rats aged 6 months was not significantly different to that observed in 24 months aged animals ($p = 0.10$).

FIGURE 7 BELONGS HERE

Figure 7. Age-dependent protein expression changes of phospholamban in the sinoatrial node.

A-D, Illustrative western blot and protein levels per age group of total PLB, alongside separated data for pentameric and monomeric forms normalised to desmin. Mean ± S.D.; $n = 5$; ANOVA. Protein levels declined in SA nodes from rats age 24 months for B monomer PLB protein levels (6 vs. 24 months, $^{*}p = 0.0016$; 12 vs. 24 months, $^{\#}p = 0.0017$), C pentamer PLB protein levels (12 vs. 24 months $^{*}p = 0.004$; 6 vs. 24 months $^{\#}p = 0.035$) and D total PLB protein levels (6 vs. 24 months $^{*}p = 0.0123$; 12 vs. 24 months $^{\#}p = 0.0120$). E,

444 The SERCA : PLB ratio protein levels increased in SA nodes from rats at 12 months of age,
445 then decline back to the 6 months rat levels (6 vs. 12 months *p = 0.0080;12 vs. 24 months
446 #p = 0.0125; 6 vs 24 months, p = 0.10).
447
448

Age dependent decline in SERCA2a activity of the SAN and pacemaker dynamics.

The IHR of the isolated SAN preparation was measured during steady-state spontaneous pacemaking under control conditions and during incrementing β-adrenergic stimulation for each of the 3 age groups. Data showed an age-associated reduction in basal intrinsic pacemaking (Figure 8A and summary in table 5) with intrinsic heart rate showing a linearly correlated reduction with age (r^2 = 0.607; p = 0.0017) falling from 274 ± 33 BPM at 6 months to 206 ± 19 BPM at 24 months of age. The EC50 to isoprenaline however remained unchanged with age (Figure 8A; 13.4 nM at 6 months and 13.7 nM at 24 months). At 1 µM isoprenaline pacemaking differences were abolished across all age groups with intrinsic rates rising to 386 ± 36 and 368 ± 62 BPM in the youngest and oldest age groups respectively, indicating that a maximal response to the highest concentration of isoprenaline remains viable even in the SAN of the oldest rats that we studied.

Table 5. Steady-state intrinsic heart rate (IHR) of isolated SAN with and without maximal β-adrenergic stimulation and/or cyclopiazonic acid (CPA) to block SERCA2A.

	6 months	**12 months**	**24 months**
IHR (beats per minute)	274 ± 33	256 ± 25	206 ± 19 *****
Maximal Rate (1 µm Iso.)	386 ± 36 #	402 ± 22 #	368 ± 62 #
IHR in CPA	216 ± 26	204 ± 9	189 ± 27
Maximal Rate in CPA (1 µm Iso.)	258 ± 30	245 ± 22	240 ± 40

***** p=0.022 comparing rate in 24-months with that at 6-months of age.

p=<0.0001 for rate in 1 µM isoprenaline vs intrinsic heart rate in same age group.

To assess the functional involvement of the SR during β-adrenergic stimulation across the age groups, 3 µM cyclopiazonic acid (CPA) was applied to inhibit SERCA2a and deplete the SR. CPA significantly reduced spontaneous activity at 6 and 12 months (p = 0.023 and 0.044 respectively; RM-ANOVA) (Figure 8A and Table 5). Interestingly, in the oldest group of 24 months of age CPA did not significantly reduce the basal intrinsic rate (p = 0.904). Intrinsic heart rate was no longer significantly different across the age groups in the presence of CPA (p = 0.307; RM-ANOVA; n = 6 in each group). CPA significantly reduced the response to isoprenaline in all age groups. In the presence of CPA the EC50 for isoprenaline did not significantly differ across age groups under control conditions or in the presence of CPA.

Activation maps for each sinoatrial node studied were constructed and the leading pacemaker site located during the steady state response to each condition. Isoprenaline caused a significant average shift in pacemaker location of 3.6 ± 1.6 mm ($p<0.0001$, n=6 in each group), Figure 8B. Application of CPA caused a small but significant shift in the leading pacemaker site (1.2 ± 0.9 mm; $p= 0.0003$) and the significant isoprenaline-dependent shift in the leading pacemaker site was maintained on subsequent application of isoprenaline in the presence of CPA in all age groups (3.78 ± 16 mm; $p<0.0001$). Comparing between age groups there was no significant difference in pacemaker shift in response to isoprenaline, CPA or the combination between age groups.

Examining the velocity of conduction between the leading pacemaker site and crista terminalis under control conditions a significant reduction in conduction velocity was seen across the SAN in the oldest group compared with tissue from the other two ages. Average conduction velocity was 4.4 ± 0.25 $m.sec^{-1}$ at 6 months; 4.6 ± 0.60 $m.sec^{-1}$ at 12 months falling significantly to 3.8 ± 0.23 $m.sec^{-1}$ at 24 months ($p = 0.041$ and 0.015 for 24 vs. 6 months and vs. 12 months respectively, n = 6 in each case).

A dose-dependent significant increase in conduction velocity in response to isoprenaline was observed (Figure 8C) in all age groups with an average increase in velocity of 3.02 ± 1.07 $m.sec^{-1}$ ($p = <0.0001$) in the presence of 1 μM isoprenaline. The magnitude of the increase in velocity in response to isoprenaline differed significantly between all age groups, with the largest increase seen in tissue from animals at 6 months of age where velocity increased by 5.1 ± 2.1 $m.sec^{-1}$ in response to 1μM isoprenaline, but 3.0 ± 1.1 $m.sec^{-1}$ for tissue from animals at 12 months and 1.9 ± 0.5 $m.sec^{-1}$ for tissue from animals at 24 months ($p < 0.0001$ for 6 vs 24 months and $p = 0.001$ for 12 vs. 24 months).

CPA did not significantly impact conduction velocity under control conditions ($p = 0.989$) but did abolished the significant impact of isoprenaline on conduction velocity in SAN tissue from all age groups with the magnitude of increase in the presence of CPA falling to 1.25 ± 0.91 $m.sec^{-1}$; $p = 0.160$). In the presence of CPA there were no significant differences between age groups in the response to isoprenaline.

Figure 8 belongs here

Figure 8. Isoprenaline-dependent response of nodal function across the age groups

514 **A**, Percentage change in intrinsic heart rate (beats per minute) (IHR) of the isolated SAN in
515 response to isoprenaline. The beating rate as determined in tissue from rats aged 6 months
516 under control conditions was taken as 100%. Upper traces show the % change (mean ±
517 S.D.) for each age group in response to incremental doses of isoprenaline. Lower traces
518 show the effects of isoprenaline in the presence of 3 μM CPA. **B**, magnitude of shift in the
519 leading pacemaker site on application of 1 μM isoprenaline, 3 μM CPA or the combination of
520 both. **C**, conduction velocity across the node from the leading pacemaker site to the crista
521 terminalis during application of different doses of isoprenaline alone or in the presence of 3
522 μM CPA.

Discussion

The decline in maximal heart rate and increasing risk of pacemaker dysfunction is a ubiquitous feature of ageing, as has been seen in many animal models and human studies (Peters *et al.*, 2020). Previous work by ourselves and others have correlated this with morphological changes of the SAN as well as changes in ion channel expression (Jones *et al.*, 2004; Jones *et al.*, 2007; Monfredi & Boyett, 2015; Moghtadaei *et al.*, 2016). Alongside this has been documented an altered response to adrenergic stimulation which may be closely linked to an impaired ability of cAMP to modulate the 'calcium clock' component of sinoatrial node pacemaking and so cause acceleration of the sinoatrial node (Liu *et al.*, 2014; Segal *et al.*, 2023). In this study we have investigated age-associated changes in key components of cellular calcium regulation on pacemaking response as well as the effect of entirely removing the SR contribution to pacemaking. The results reveal a sinoatrial node in the elderly which operates at an intrinsically lower spontaneous frequency, but when the calcium clock is disrupted age-associated differences in pacing rate and adrenergic response are removed. The detailed mapping of protein changes and functional adaptations identify a largely balanced scaling of changes with age preserving function and responsiveness in the node but ultimately also show dysregulation of the 'calcium clock' may underlie key age-associated changes in functional capacity, tolerance and response.

Right atria and ageing

Our male Wistar rat model showed that post-maturity with increasing age their body weight increased significantly, as expected, but the heart weight did not change significantly with age. Previous work in rats and other species, including humans have reported either no change in heart weight simply with age, (Hindso *et al.*, 2017) or more commonly, age-associated hypertrophy that may be secondary to hypertension and body mass increases with age (Linzbach & Akuamoa-Boateng, 1973). Other studies have even indicated potential reductions in cardiac mass relative to lean body mass in old age (Olivetti *et al.*, 1991), but essentially it is the metabolically active tissue that is expected to be the key driver of cardiac output.

Across our studies we have used the muscle-specific protein desmin to normalise our western blot data, (Jones *et al.*, 2004; Jones *et al.*, 2007) as both mRNA and protein levels of desmin do not change with age as shown in the rat atria (Table 4), whereas the metabolically active protein Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) showed significant changes in levels of mRNA with increasing age within the RA (Table 3). Our initial studies of calcium ion channels within the rat RA showed an age-associated significant

increase of $Ca_v1.3$ and $Ca_v3.1$ protein levels, and a significant decline in $Ca_v1.2$, whilst $Ca_v1.2$ did not alter with age in the LA (Figures 2 & 3). Our key focus however has been the SAN within the RA and age-related changes in calcium regulation that may be preponderant there.

Age-dependent changes in calcium regulators

We show a significant loss of $Ca_v1.2$ channel protein from within the SAN of the rat during advancing age. Such a change will potentially result in reduced excitability of the cells of the node as well as reduced calcium-loading of the SR, potentially limiting the ability of the SR to modulate pacemaking via the calcium clock. These channels are key for the upstroke of the action potential in SAN myocytes once threshold is reached but also serve to service the calcium loading of the cell, thus directly modulate functioning of the 'calcium clock' as well as the funny current I_f indirectly (Hagiwara & Irisawa, 1989). Of considerable interest is the observed shift in association between pharmacological modulation of the current through $Ca_v1.2$ using nifedipine and the ensuing impact on heart rate. Whilst the EC50 does not shift, implying the interaction with the channel in terms of binding is conserved, the four-fold shift on the Hill coefficient indicates a notable change in the relationship between the current and pacemaking. The elevated sensitivity may in part be simply due to the overall drop in channel density but also reflects a shift in the links between membrane calcium flux via $Ca_v1.2$ and pacemaking. The relationship between pacemaking and this flux is complex to assess since this acts as a depolarising influence but also critically a key loading flux for the SR, which we see is also altering in influence in old age, together with other elements that ensure calcium balance such as NCX. The steep sensitivity implies a reduced capacity to deal with flux variation and still maintain calcium balance within a suitable range to ensure continued stable pacemaking. In the face of an already reduced inward calcium flux and in tandem with reduced SR function the node experiences a steep reduction in potential depolarising current from both the calcium current and SR calcium releases, with the net impact observed here.

Age and changes in the balance of 'calcium clock' components

Analysing the components of the SAN calcium clock we see broad depression in the expression of all elements associated directly with the sarcoplasmic reticulum and its function. Calcium release and uptake proteins are reduced by a similar magnitude in old age indicating possible co-regulation to ensure maintained equilibria essential for continued stable function, since cumulative imbalance of calcium flux will rapidly lead to arrhythmias or cell death. SERCA2a is key for maintaining loading of the SR with calcium and is a key controller of diastolic calcium in partnership with calcium entry via the L-type calcium

channel. Modulating this relationship is the influx of calcium, and the activity of SERCA2 modulated by PLB in response to production of cAMP triggered by adrenergic stimulation (Capel & Terrar, 2015). SERCA, RYR and PLB all fell proportionately in old age maintaining this control equilibrium and perhaps more indicative of a general loss in SR density rather than shifting control. As a potential means to ensure maintained calcium balance, even though L-type calcium channel expression is also falling, NCX rises. As such in old age the scope for calcium release from the SR to dynamically respond to pacemaking is likely to be suppressed, although the elevated NCX may be sufficient to facilitate the basal contribution of depolarising current secondary to SR release (Groenke *et al.*, 2013) which is potentially occurring at a reduced density. Previous work in an over-expressor model of NCX showed elevations in NCX to be capable of increasing the dynamic response to adrenergic stimulation (Kaese *et al.*, 2017). Here we do not see such an enhancement but the elevation in NCX is certainly likely to be key in maintaining the response we do see in the face of a falling potential SR component driving pacemaking.

Impact of stopping the 'calcium clock' leaving the membrane one to run the beat

The key role of the calcium clock in regulating the SAN is seen when we effectively remove the function of the SR using CPA to inhibit SERCA function. This only impacted basal pacemaking of the SAN significantly in tissue from the younger groups. In contrast, CPA had insignificant impact on SAN activity in tissue from elderly animals indicating a key shift in the influence of the calcium clock on activity of the node. One surprising observation is that despite the apparent lack of a role for the SR in maintaining the pacemaker rate at baseline in the old animals they do show an apparently normal dose-response to isoprenaline with a maintained EC50, and this response remains comparable between age groups in the presence of CPA. The implication is that the signal transduction processes responsible for the adrenergic response remain perfectly functional in old age. We haven't looked in detail at each part of the signal transduction process, but other studies have similarly found preserved potential for response (Liu *et al.*, 2014) even though kinetics and sensitivity may be subtly altered at various stages of the process from catecholamine interaction with receptors through to cAMP generation (Xiao *et al.*, 1998) and equilibrium with phosphodiesterases (Yaniv *et al.*, 2016). Despite this preserved potential for response in the isolated SAN, maximal heart rate in the intact organism is depressed with age indicating key aspects such as neural input and metabolic regulation which may be differentially modulated or limited in the intact organism may be key for developing our understanding of why peak heart rate is supressed in the elderly.

A key observation is that with SR function supressed the response to isoprenaline at all ages becomes equivalent, although much depressed in range. Thus, it seems in the male rat at least, modulation of If by cAMP is insufficient alone to drive a normal full pacemaker response to adrenergic input. The ~70% reduction in dynamic response to isoprenaline seen in the SAN with SERCA inhibition present suggests, in the rat at least, activation of HCN and the membrane clock can only achieve ~30% of the normal increase in activation frequency. This balance in dynamic response seems preserved across the age range even though basal activity is suppressed in SAN tissue from the oldest animals we studied again implying balanced regulation across the age range rather than identifying a distinct imbalance arising that can be targeted to reduce the impact of ageing.

Impact on pacemaker response and conduction

We have conducted our experiments using the intact functioning SAN. A key regulator of pacemaker activity isn't just seen at the individual cell level since the activation of the node and it's ability to drive the pacing of the heart is dependent on the electrotonic interactions between cells of the leading pacemaker site and surrounding tissue. Changes in pacemaker frequency are commonly associated with shifts in dominant pacemaker location and propagation of activation across the tissue. At it's most extreme there are suggestions that in some species, including humans, distinct foci for pacemaking depending on frequency may exist (Brennan *et al.*, 2020) representing areas with differing expression of ion channels and/or electrotonic interaction with the surrounding tissue modulated by frequency and focussed autonomic influence. In the isolated nodal preparations, we have used we do not have focussed autonomic input, but we did still see significant shifts in the location of the leading pacemaker site when isoprenaline was applied, even when SR function was inhibited with CPA. This shift was preserved across all age groups. Despite a significant shift in basal pacemaker frequency in tissue from the younger ages studied, no shift in pacemaker location was however observed when SR function was inhibited with CPA. This observation raises questions regarding what may drive pacemaker shift. If shifts in calcium balance and even depolarisation frequency, of the magnitudes we have seen with age and CPA are not enough to cause pacemaker shift then maybe this may represent simply a difference in density of expression of adrenergic receptors or other elements of the transduction machinery for actioning the adrenergic response. A surprising observation was the shift in conduction velocity across the node with isoprenaline. An increase in conduction velocity is expected as the upstroke velocity and amplitude of the action potential is increased with adrenergic stimulation, but this was only observed in nodal tissue from the younger animals. The failure of isoprenaline to be able to increase conduction velocity in the oldest group may be indicative of the loss of calcium channels impacting the upstroke of the

action potential, or equally may reflect loss of connexins key to permit rapid electrical propagation (Jones *et al.*, 2001). Irrespective of the reason, this data indicates a limitation for supporting stable higher-frequency activation of the node and surrounding tissue due to limits in the rate at which the activation can spread to the atria. This has the potential to be a key issue for the development of sick-sinus syndrome and atrial fibrillation, although future work making more-detailed sub-regional analysis all across the node is required to clearly identify how this failure of conduction is arising.

Clinical Implications and Implications for Human Ageing

The identified shifts in calcium regulatory processes, baseline pacemaker rate as well as suppressed ability to enhance conduction velocity are all key issues to consider in the perspective of the aged human heart. Calcium channel blockers such as verapamil are still used widely, chiefly for their impact on vascular function but the changing relationship between calcium balance and pacemaker function does indicate a developing danger. Indeed use of verapamil in patients with sick sinus syndrome has previously been noticed to have four to five times the impact on SAN function as when applied in control individuals, a comparable magnitude of shift as our identified shift in slope of the response of the isolated rat SAN to calcium channel block (Carrasco *et al.*, 1978). The obvious clinical implication of this is to use calcium channel modulators with considerable caution in the elderly, paying careful attention to the potential for inducing and exacerbating sick-sinus syndrome.

In the light of broader human ageing the implications remain that until we can solve the issue of the continued drop in SAN function and peak heart rate with age we cannot expect to be able to significantly prolong maximal lifespan without encountering pacemaker problems. Solutions do not appear to simply lie in restoring response to adrenergic stimulation, but instead are likely to require maintenance or restoration of key aspects determining conduction though the SAN and capacity within the calcium regulatory processes, membrane and SR associated, to ensure stable response and continued activity at suitable rates to meet requirements for adequate cardiac output.

Limitations and Future Work

One interesting feature of our findings was we did not see a significantly limited response to isoprenaline in aged tissue as generally reported by others in the intact heart and other preparations (Lakatta *et al.*, 1975). In other cases, what has often been seen is a shift in the dose-response curve to adrenergic stimuli whilst direct elevations of cAMP or saturating doses of agonists show maximal responses are in fact preserved (as seen here and for example by Liu et al. (Liu *et al.*, 2014). As such *in-vivo* a depressed maximal rate is

common, but ex-vivo any differences in signal transduction arising from alterations to autonomic input or accumulation of agonists it would appear can be negated. The implication is that the end effector of heart rate elevation the peak rate of nodal depolarisation remains capable of a full range of function in advanced age, so to identify the source of depressed response we need to look to manipulate the signal transduction pathway if we wish to restore youthful function. Having said this, we do see signs of potential for greater instability due to the changing balance of components regulating intracellular calcium buffering. Future experiments will aim to assess this more directly but also identify steps on the signalling pathway that alter the relationship between cAMP production and activation of the integrated pacemaking mechanism of the calcium and membrane clocks in advanced age. A key feature to permit stable restoration of higher pacemaker rats to the aged SAN though would also appear to be modulation of conduction.

One limitation in translating these findings to the human condition is understanding that the shifts in impact of differing mechanisms of pacemaker modulation and atrial excitability will vary between species and also ages. For example, in humans, the resting heart rate is normally kept reduced by parasympathetic input and on removing this and observing the intrinsic rhythm an acceleration is seen (Christou & Seals, 2008). In others, such as the rat as we have studied here, the resting heart rate *in vivo* is maintained by tonic sympathetic input and on removing this the intrinsic rate is, in contrast to humans, lower than that seen *in vivo* (Di Gennaro *et al.*, 1987). This latter response may also apply to the aged human heart (Christou & Seals, 2008), potentially making the rat heart more in-line with the aged human heart. These indicate a differing role for tonic sympathetic drive across ages and species which will go hand-in-hand with associated signal transduction, response and the role of pacemaker mechanisms in facilitating the dynamic response of the heartbeat. Further experiments on human tissue are required to refine our understanding of the relative impact of the membrane and calcium clocks across the SAN and how each are modulated by adrenergic stimulation and age.

Conclusion

In conclusion, our data shows a changing relationship between pacemaker function and cellular calcium handling with advancing age. Whilst the identified changes also show preservation of a good level of calcium handling function and response to isoprenaline, there are indicators of an increased risk of instability and altered responses to physiological and pharmacological interventions. Pharmacological agents modulating the changing dynamics driving the SAN activity are routinely administered in treatment of hypertension for example,

however due to an increasing risk of precipitating sinus node dysfunction greater care is needed in consideration of their use in elderly patients.

Additional Information

All authors declare there are no competing interests or conflicts of interest.

AI was not used to generate any content for this manuscript.

Drs Godbeer, Jones and Lancaster contributed to the data collection process and analysis. Manuscript drafting, editing and further data analysis was conducted by Drs Jones and Lancaster.

Data Availability Statement: Raw data for activity responses shown in Figures 4 and 8 are available as online Supporting Information.

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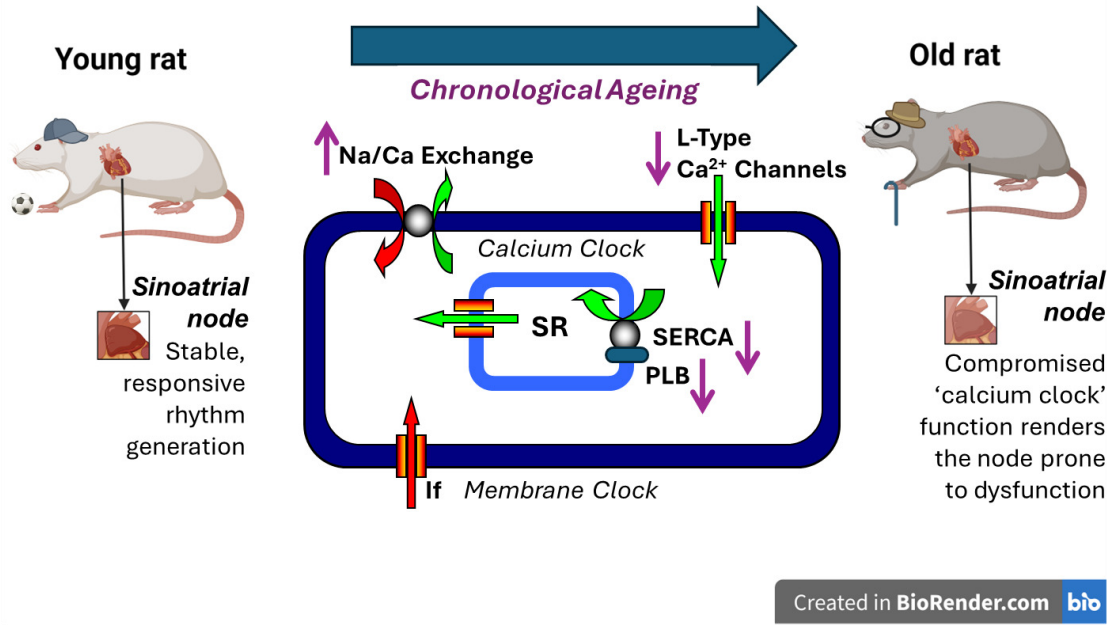

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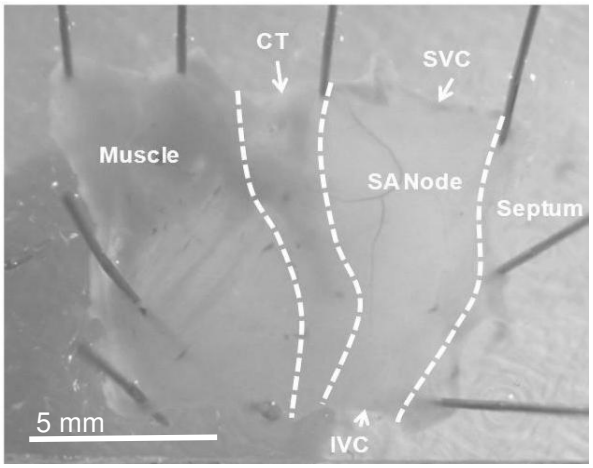


Figure 1. Endocardial surface of the right atria

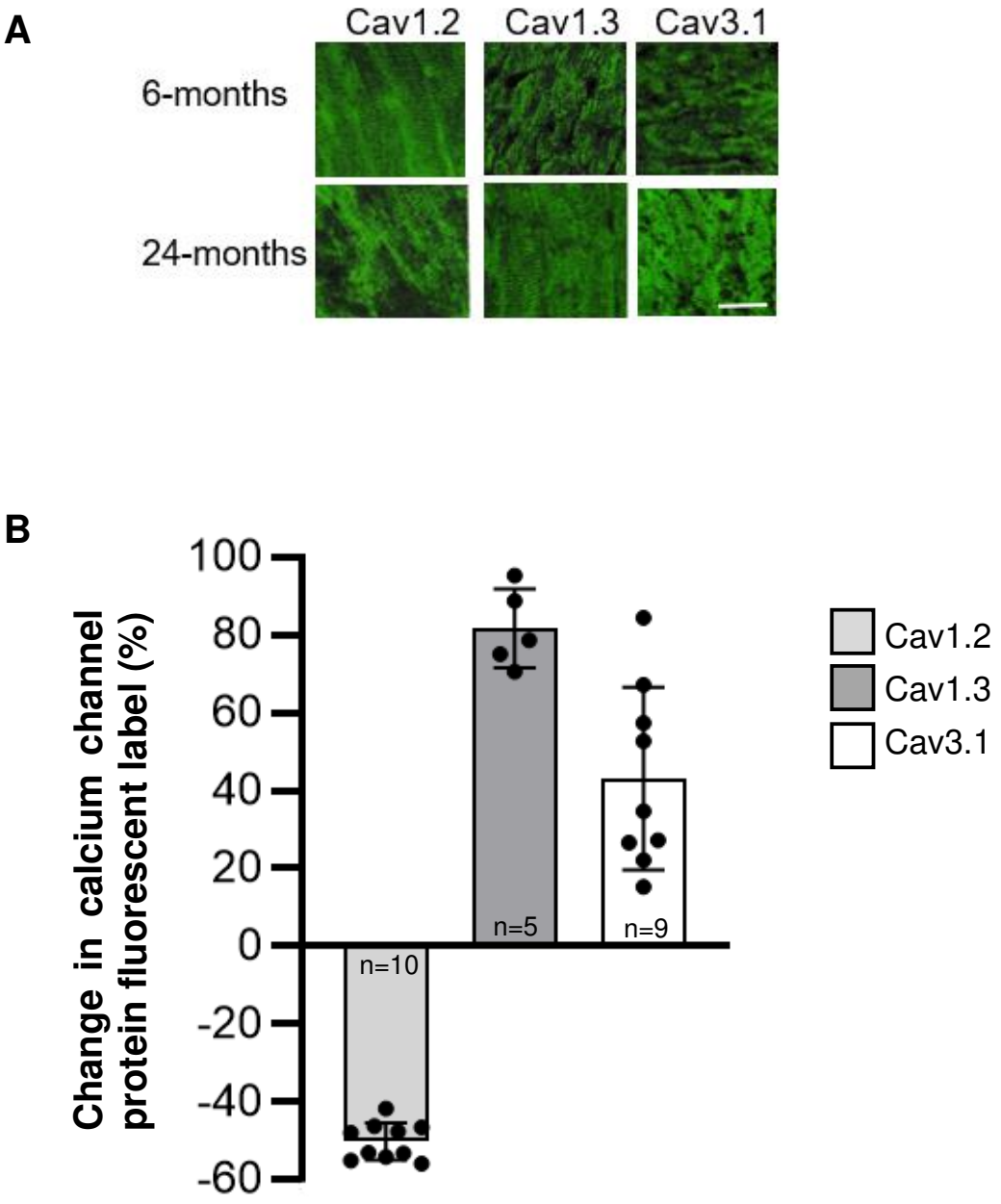


Figure 2. Changes in IHC calcium channel label within RA at 24-months of age.

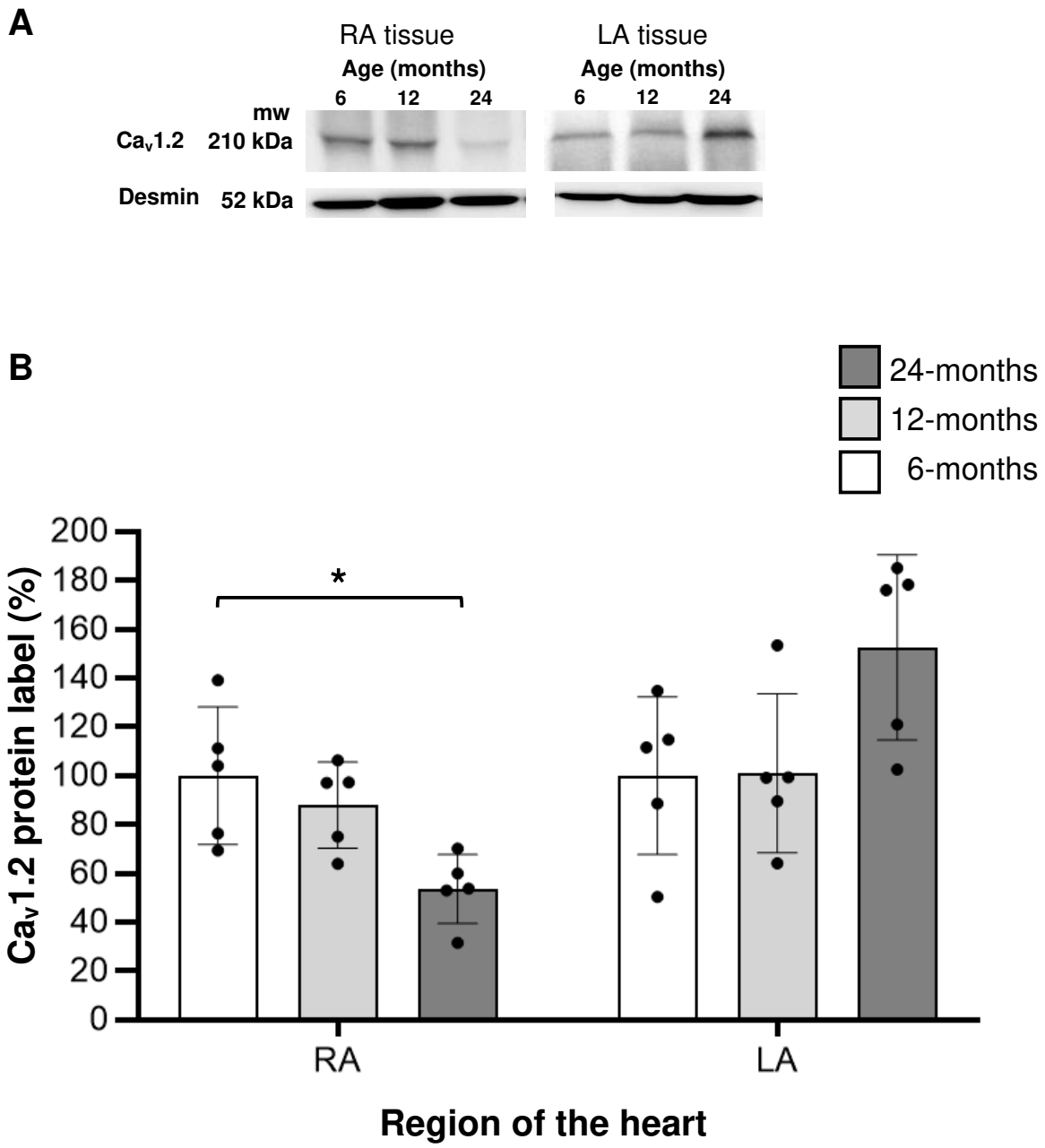


Figure 3. Age-associated changes of $Ca_v1.2$ protein across the atria.

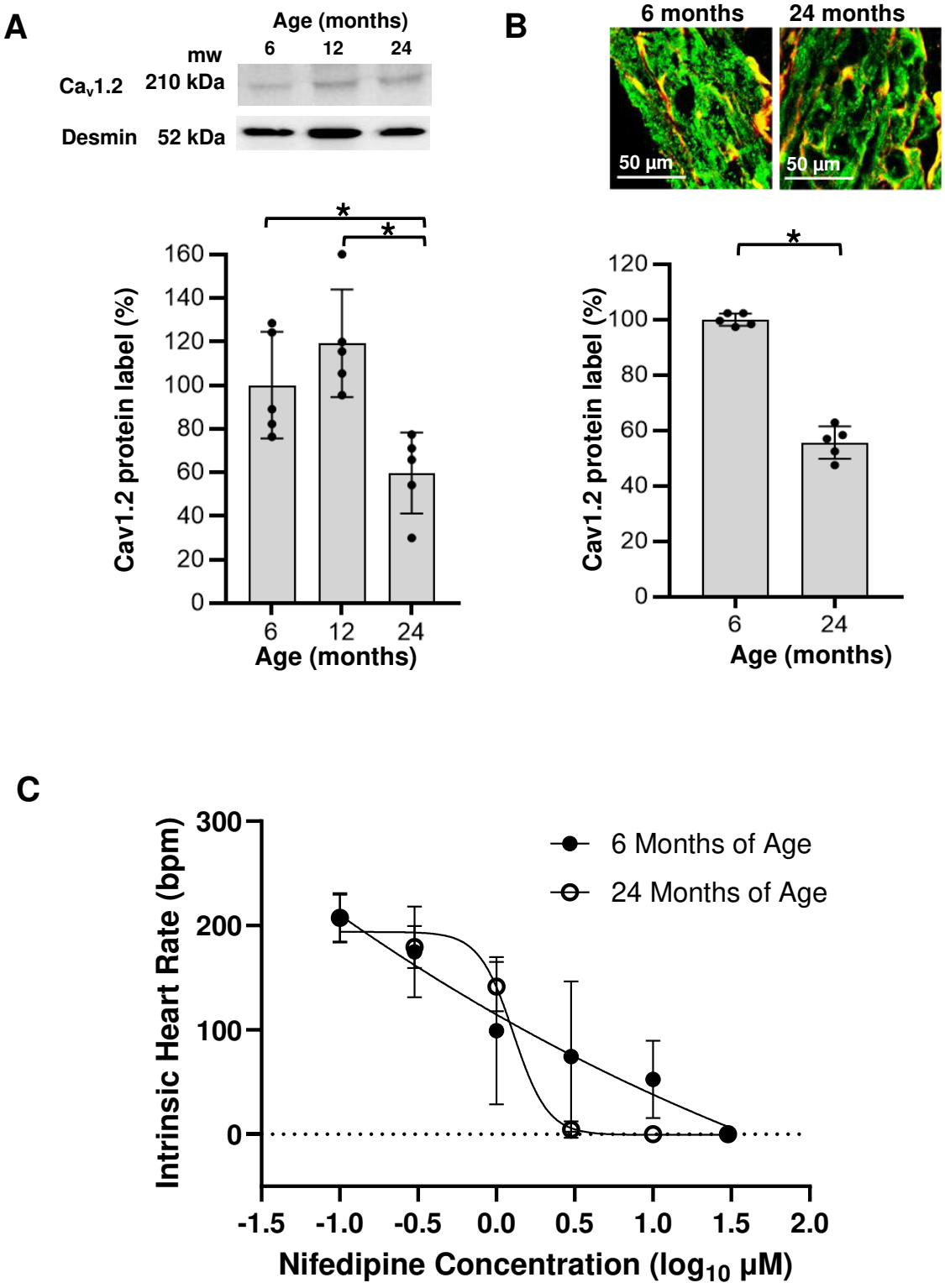


Figure 4. Age-associated changes of $Ca_v1.2$ protein and activity within the SAN.

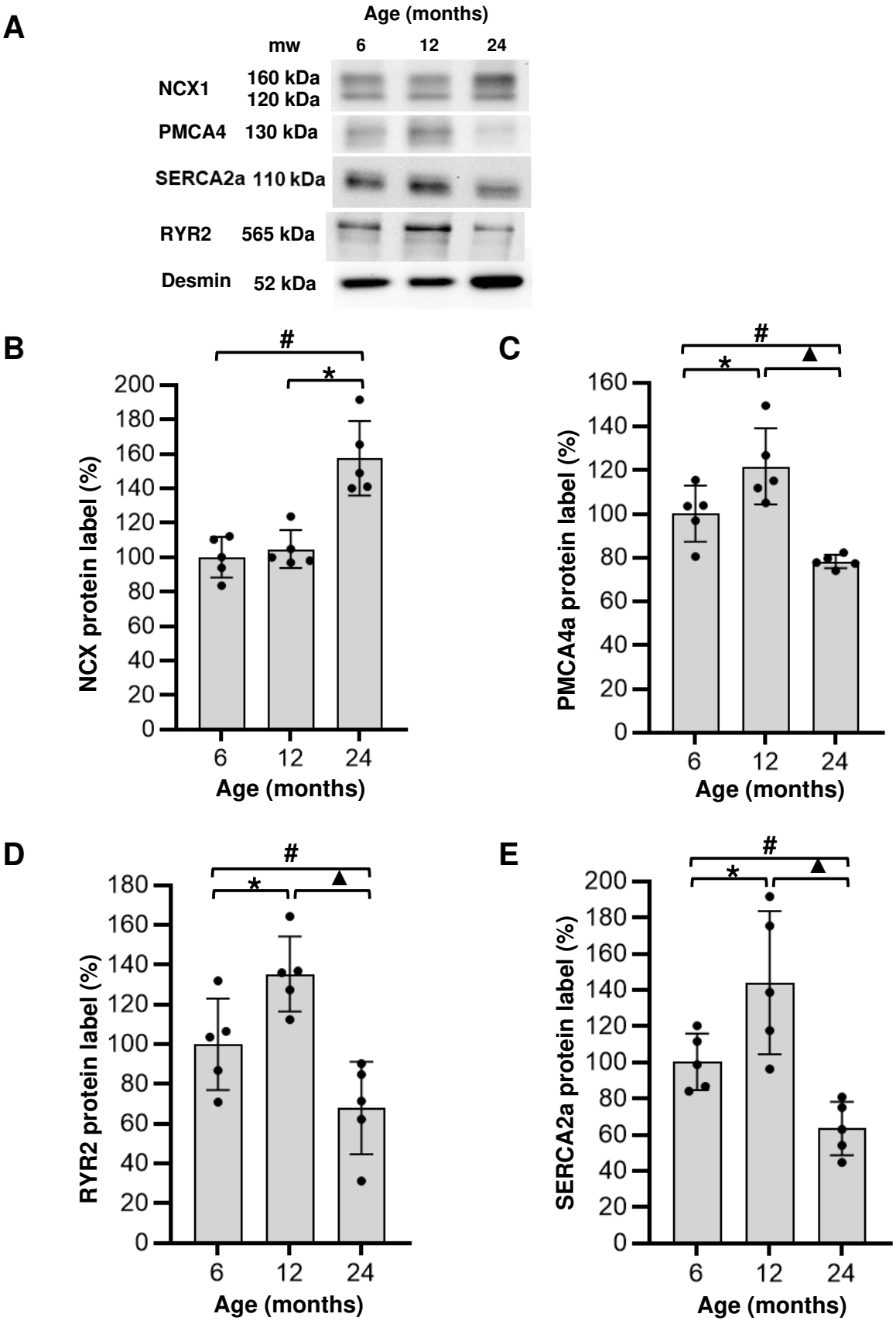
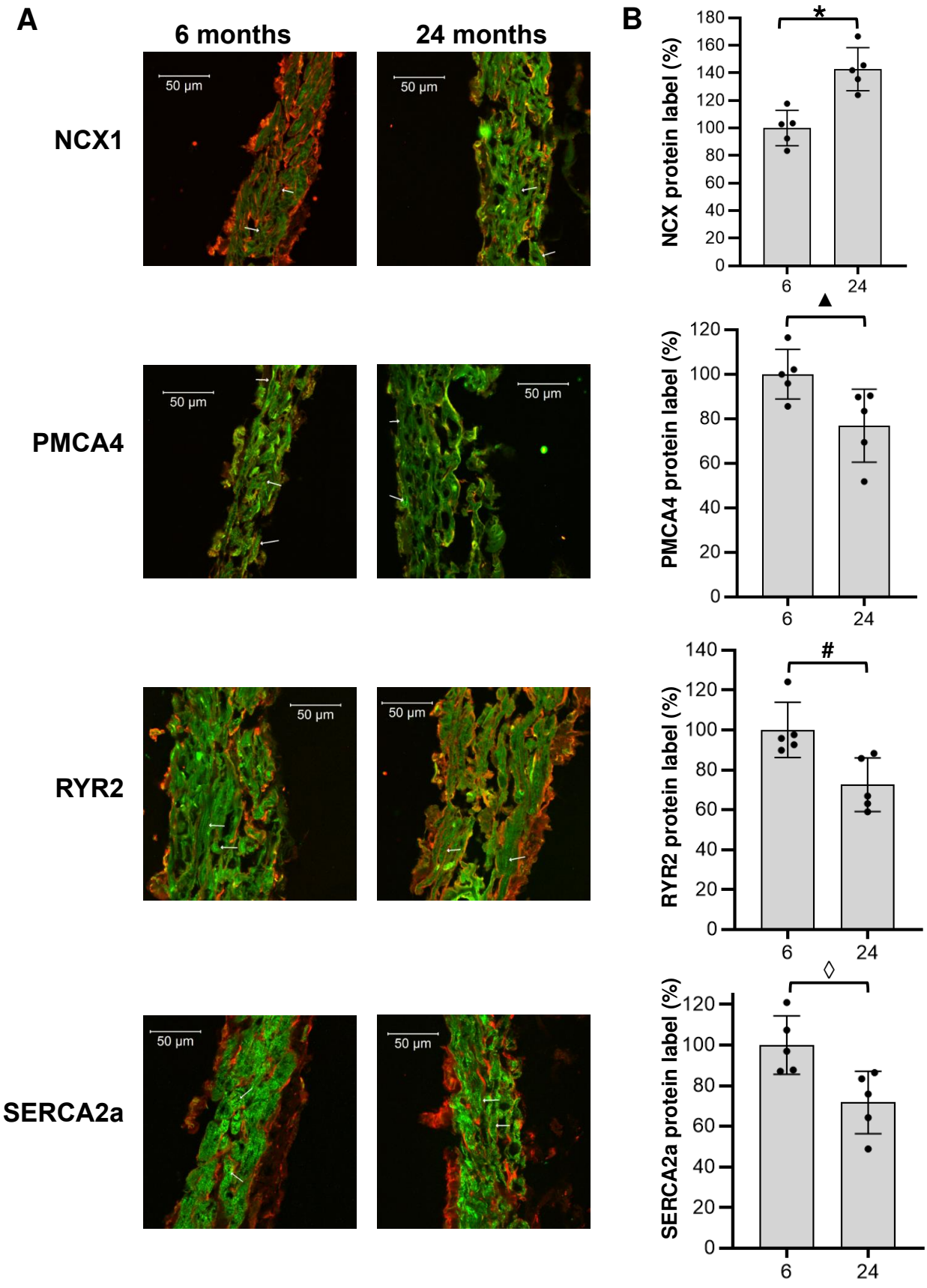


Figure 5. Effect of ageing on levels of calcium handling proteins expressed in the sinoatrial node.



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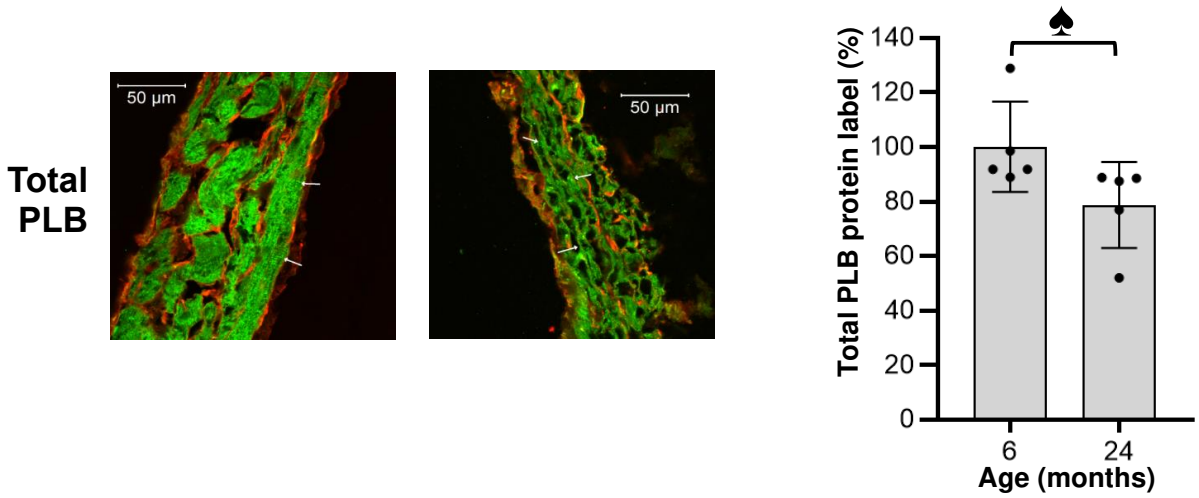

Figure 6. Age-dependent changes of proteins labelled within the sinoatrial region.

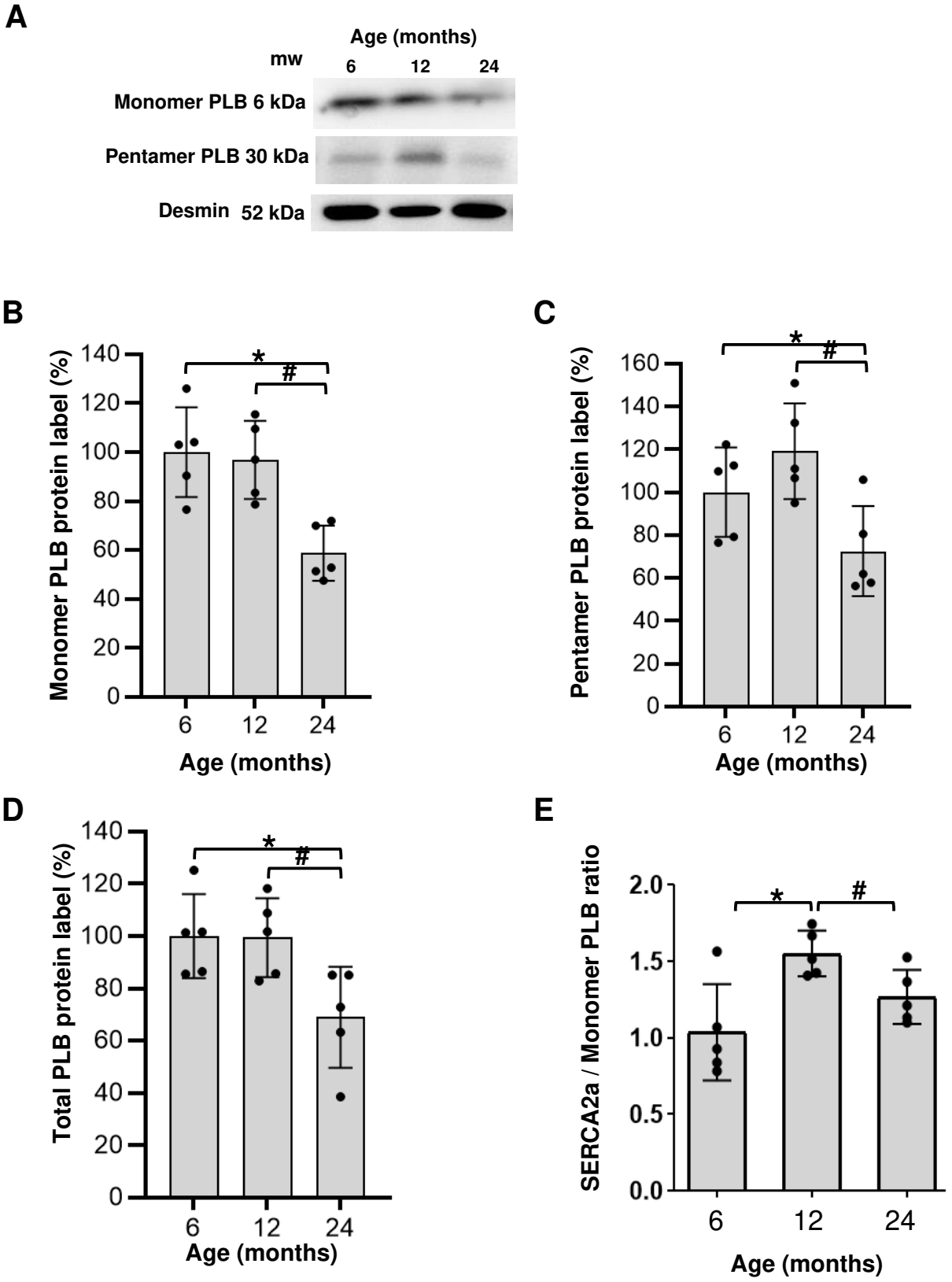

Figure 7. Age-dependent protein expression changes of phospholamban in the sinoatrial node.

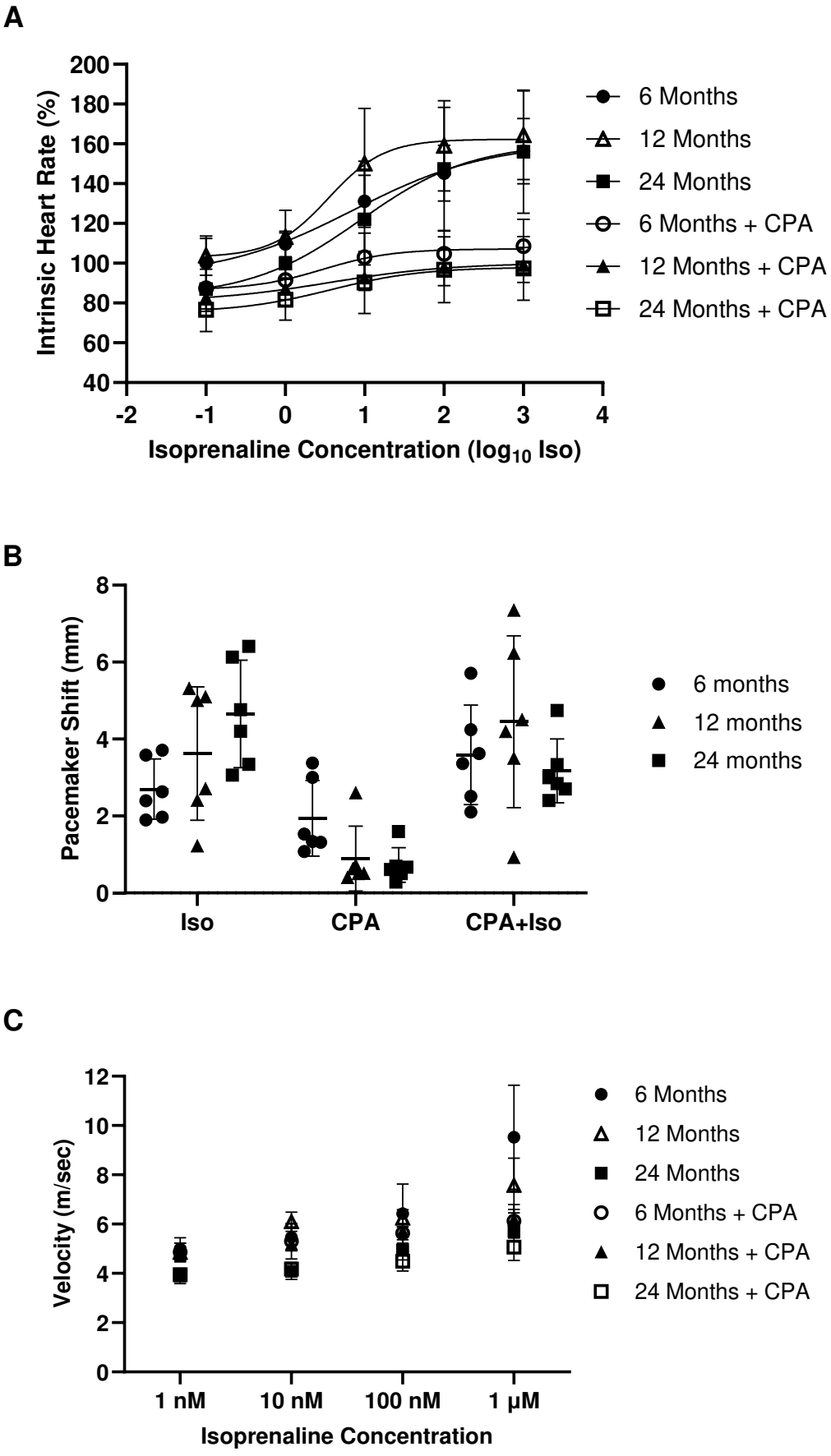

Figure 8. Isoprenaline-dependent response of nodal function across the age groups