Increasing T-type calcium channel activity by β-adrenergic stimulation contributes to β-adrenergic regulation of heart rates

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Key points
• Cav3.1 T-type Ca\(^{2+}\) channel current (I\(_{\text{Ca-T}}\)) contributes to heart rate genesis but is not known to contribute to heart rate regulation by the sympathetic/β-adrenergic system (SAS).
• We show that the loss of Cav3.1 makes the beating rates of the heart in vivo and perfused hearts ex vivo, as well as sinoatrial node cells, less sensitive to β-adrenergic stimulation; it also renders less conduction acceleration through the atrioventricular node by β-adrenergic stimulation. Increasing Cav3.1 in cardiomyocytes has the opposite effects.
• I\(_{\text{Ca-T}}\) in sinoatrial nodal cells can be upregulated by β-adrenergic stimulation.
• The results of the present study add a new contribution to heart rate regulation by the SAS system and provide potential new mechanisms for the dysregulation of heart rate and conduction by the SAS in the heart.
• T-type Ca\(^{2+}\) channel can be a target for heart disease treatments that aim to slow down the heart rate

Abstract Cav3.1 (\(\alpha_{1C}\)) T-type Ca\(^{2+}\) channel (TTCC) is expressed in mouse sinoatrial node cells (SANCs) and atrioventricular (AV) nodal cells and contributes to heart rate (HR) genesis and AV conduction. However, its role in HR regulation and AV conduction acceleration by the

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Yingxin Li received his Ph.D. degree from Temple University, where he focused on ion channel electrophysiology. He found that upregulating T-type calcium channel activity contributes to the regulation of heart rates. This led him to studying the electrophysiology of cardiomyocytes derived from patient-specific human induced pluripotent stem cells, which can serve as a platform for precision medicine and drug toxicity evaluations. His career goal is to accelerate drug discovery and development and to implement precision medicine. Xiaoxiao Zhang graduated from the Fourth Military Medical University with an MD degree in 2010 and then joined the department of Ultrasonography of Union Hospital affiliated with Huazhong University of Science and Technology (HUST). From 2013 to 2016, she was trained as a postdoctoral fellow in Temple University and then obtained her PhD degree in 2017 from HUST. During her work and study, she developed strong interest in cardiac research.

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\( \beta \)-adrenergic system (SAS) is unclear. In the present study, L- (\( I_{Ca-L} \)) and T-type (\( I_{Ca-T} \)) Ca\(^{2+} \) currents were recorded in SACs from Cav3.1 transgenic (TG) and knockout (KO), and control mice. \( I_{Ca-L} \) was absent in KO SACs but enhanced in TG SACs. In anaesthetized animals, different doses of isoproterenol (ISO) were infused via the jugular vein and the HR was recorded. The EC\(_{50} \) of the HR response to ISO was lower in TG mice but higher in KO mice, and the maximal percentage of HR increase by ISO was greater in TG mice but less in KO mice. In Langendorff-perfused hearts, ISO increased HR and shortened PR intervals to a greater extent in TG but to a less extent in KO hearts. KO SACs had significantly slower spontaneous beating rates than control SACs before and after ISO; TG SACs had similar basal beating rates as control SACs probably as a result of decreased \( I_{Ca-L} \) but a greater response to ISO than control SACs. \( I_{Ca-T} \) in SACs was significantly increased by ISO. \( I_{Ca-T} \) upregulation by \( \beta \)-adrenergic stimulation contributes to HR and conduction regulation by the SAS. TTCC can be a target for slowing the HR.

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### Introduction

Cardiac rhythm (heart rate, HR) generation and regulation are important for the function of the heart. HR is dictated by the automaticity of sinoatrial node (SAN) cells, which is controlled via a complex interplay of membrane ion channels, transporters and intracellular Ca\(^{2+} \) (Nargeot & Mangoni, 2006; Jaleel et al. 2008; Shinohara et al. 2010; Lau et al. 2011). To adapt to the needs of the body, the HR of animals and human beings is constantly regulated (Lau et al. 2011). HR regulation occurs by a change in the automaticity of the SAN cells and the conduction rate, especially via the atrioventricular node (AVN) in response to changes in autonomic nerve activity (Lakatta et al. 2010). The increases in sympathetic outflow to the SAN and AVN with concurrent inhibition of vagal tone lead to an increase in HR. The excitation of the sympathetic/\( \beta \)-adrenergic system (SAS) releases catecholamines that bind to \( \beta \)-adrenergic receptors and activate cAMP/protein kinase A (PKA) signalling (Alig et al. 2009). The major type of \( \beta \)-adrenergic receptors mediating the regulation of SAN automaticity are the \( \beta 1 \)- and \( \beta 2 \)-adrenergic receptors (Rohrer et al. 1999).

CAMP increases depolarizing funny current (\( I_f \)) directly and indirectly by PKA (‘membrane clock’) to augment the HR (Lakatta et al. 2010). It has been proposed that the enhancement of sinoatrial node cell (SAN) intracellular calcium cycling by rhythmic activation of PKA (‘calcium clock’) also contributes to SAS-mediated upregulation of HR (Vinogradova & Lakatta, 2009). Both mechanisms contribute to an increased slope of diastolic depolarization (phase 4 depolarization) so that the pacemaker potential reaches the threshold for firing more rapidly. On the other hand, parasympathetic activation releases acetylcholine (Ach) onto the SAN to activate Gi, which decreases intracellular CAMP and activates hyperpolarizing K\(_{ach} \) channels, decreasing the membrane potential and the slope of diastolic depolarization of SACs, leading to a slower HR (Nishi et al. 1976; Kaspar & Pelzer, 1995; Kondratyev et al. 2007). Abnormal SAN automaticity and its regulation contribute to sinus node disease, a type of arrhythmia often requiring pacemaker implantation (Lau et al. 2011), whereas modulating SAN automaticity is an effective way of treating heart failure (Borer et al. 2016).

Other depolarizing currents such as T-type Ca\(^{2+} \) currents (\( I_{Ca-T} \)) also contribute to the ‘diastolic depolarization’ essential for SAN pacemaking (Mesirca et al. 2015). T-type Ca\(^{2+} \) channels (TTCCs) are a family of voltage-gated Ca\(^{2+} \) channels with ‘tiny’ single-channel conductance and fast decay rates (‘transient’) (Perez-Reyes, 2003; Nilius et al. 2006). These channels are activated at low membrane potential thresholds (~70 mV to ~−60 mV) (Perez-Reyes, 2003; Nilius et al. 2006), enabling them to participate in diastolic depolarization of pacemakers. There are three subtypes of TTCCs encoded by three genes: \( \alpha 1 \) subunits, \( \alpha 1G \) (Cav3.1), \( \alpha 1H \) (Cav3.2) or \( \alpha 1I \) (Cav3.3) (Perez-Reyes, 2003; Nilius et al. 2006). In mammalian hearts, Cav3.1 and Cav3.2 are expressed in the whole heart during the embryonic stage, although their expression in ventricles decreases rapidly after birth (Cribbs, 2010). Cav3.1 and Cav3.2 expression remains in the SAN and AVN cells of the adult heart, indicating a role of Cav3 channels in automaticity (Mangoni & Nargeot, 2001; Efimov et al. 2004; Ono & Iijima, 2010). \( I_{Ca-T} \) has been recorded in adult SACs from multiple species (Hagiwara et al. 1988; Zhang et al. 1988).
et al. 2003; Mangoni et al. 2006). Cav3.1 deficiency in mice slows AV conduction and the intrinsic HR recorded with blockers for both parasympathetic and sympathetic nervous systems, and also prolongs the SAN recovery time (Mangoni et al. 2006). By contrast, mice with α1H deletion have normal sinoatrial rhythm (Chen et al. 2003), in accordance with our previous findings showing that Cav3.1 mRNA but not Cav3.2 mRNA is abundant in mouse SAN cells and that Cav3.2 knockout (KO) mouse SAN cells had intact \( I_{Ca-T} \) (Li et al. 2012). Cav3.1 mRNA is abundantly expressed in human SA node (Chandler et al. 2009). Therefore, TTCCs may contribute to pacemaking in SANCs (Irisawa & Hagiwara, 1988; Huser et al. 2000).

We previously reported that Cav3.1 T-type calcium current \( (I_{Ca-T}) \) in mouse ventricular myocytes can be upregulated by β-adrenergic stimulation via a PKA-dependent mechanism (Li et al. 2012). However, the roles of this upregulation of TTCC activity in HR regulation by the SAS have not been investigated. In the present study, we hypothesize that Cav3.1 plays an important role in SAS-mediated HR regulation. To test our hypothesis, mice with inducible and cardiomyocyte-specific overexpression (gain of function, transgenic, TG) or with the KO (i.e. loss of function) of Cav3.1 were used. The findings of the present study suggest that the Cav3.1 T-type \( \text{Ca}^{2+} \) channel is an important player in HR regulation by the SAS.

**Methods**

**Ethical approval**

The present study conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Institutional Animal Use and Care Committee at Temple University. The experimental procedures were also conformed to the principles and regulations as described by Grundy (Grundy, 2015).

**Mouse models**

A TG mouse model with cardiac-specific [i.e. driven by α-myosin heavy chain (α-MHC) promoter] and inducible (tet-off, controlled by doxycycline) overexpression of mouse Cav3.1 (Genebank: NM_009783) was established with the bi-transgenic system initially in the FVB genetic background (Sanbe et al. 2003; Li et al. 2012) and then transferred to the c57bl/6 background by breeding with c57bl/6 wild-type mice for six generations. Mice were bred and genotyped as described previously (Li et al. 2012). Doxycycline-containing chow was removed after weaning to allow Cav3.1 transgene to be expressed. Cav3.1 KO mice in c57bl/6 genetic background were obtained from the Nakayama et al. (2009). C57bl/6 wild-type mice were used as the control. Both sexes of animals in equal amount were used at the age of 4 months in the present study. α-MHC promoter-driven green fluorescent protein (GFP) transgenic (non-inducible) mice were obtained from the Jackson Laboratories (Bar Harbor, ME, USA).

**ECG and intra-left ventricular pressure recording before and after isoproterenol (ISO) infusion via jugular vein in anaesthetized mice**

Mice were anaesthetized with sodium phenobarbital \([50 \text{ mg kg}^{-1} \text{ body weight} (\text{BW}), \text{i.p.}]\). Rectal temperature was continuously monitored and maintained at 37°C using a heating pad. Standard three lead ECGs were recorded using three tungsten-coated needle electrodes inserted s.c. into the appropriate forelimbs and hindlimbs with a Powerlab system (ADI, Denver, CO, USA). Intra-left ventricular pressure was recorded with a 1.4 Fr Millar pressure catheter that went into the left ventricle through the right carotid artery (Tang et al. 2010). Intra-left ventricular pressure and ECGs were recorded before (baseline) and after the application of a β-AR agonist, ISO. Different doses of ISO \([10^{-16} \text{ g} \sim 10^{-6} \text{ g} \text{ g}^{-1}]\) were applied through a jugular vein catheter as described previously (Li et al. 2014).

Because ISO stimulates all three types of β-adrenergic receptors, we aimed to determine which type of β-adrenergic receptor is involved in regulating Cav3.1 TTCC and the related HR change. Because the literature suggests that β1- and β2-adrenergic receptors are the major types of adrenergic receptors in HR regulation (Rohrer et al. 1999; Barbuti & DiFrancesco, 2008), we focused the present study on a β1-AR antagonist, metoprolol and a β2-AR antagonist, ICI 118,551. After anaesthetizing the mice with sodium phenobarbital \( (50 \text{ mg kg}^{-1} \text{ BW}, \text{i.p.}) \), the animal was taped on a heating pad and attached to three ECG leads as described above. Surface ECG was recorded for 4 min per segment at baseline and after the application of three doses of ISO \([10^{-10}, 10^{-9} \text{ and } 10^{-8} \text{ g} \text{ g}^{-1} \text{ BW, i.p.}]\) with the Powerlab and LabChart system (AD1). Next, the animals were allowed to recover for 1 week and the surface ECG was recorded after pretreatment with ICI 118,551 \( (2 \times 10^{-3} \text{ g g}^{-1} \text{ BW}) \) for 5 min and after three serial doses of ISO. After an additional 1 week, the animals were pretreated with ICI 118,551 plus metoprolol \( (2 \times 10^{-3} \text{ g g}^{-1} \text{ BW}) \) and then ECGs were recorded at baseline or after ISO application.

**Intra-right atrial pacing and recording of cardiac electrical activity**

Mice were anaesthetized with sodium phenobarbital \( (50 \text{ mg kg}^{-1} \text{ BW, i.p.}) \) and placed on a heating pad in the supine position. Rectal temperature was continuously monitored and maintained at 37°C. Standard three lead
ECGs were recorded with the Powerlab system (ADI). At the same time, an eight-lead pacing/recording catheter (EPR 800; Millar, Houston, TX, USA) was inserted into the right jugular vein and carefully advanced into the right atrial so that a strong atrial electrogram and a weaker ventricular electrogram could be recorded simultaneously. Once the pacing catheter was at the right position, it was tied with the jugular vein to fix the position. Then, programmed stimulation of the atria was applied with programmed pacing protocol generated by LabChart (ADI). The stimulation frequency was set 600 Hz and the optimal pacing amplitude was determined for each mouse to ensure each pacing was followed by the activation of the ventricles (i.e. followed by QRS waves in the surface ECG).

ECG recording from Langendorff-perfused isolated hearts

Animals were killed with sodium pentobarbital (120 mg kg⁻¹ BW, i.p.) and hearts were excised. Hearts were then hung onto a cannula connected to a Langendorff apparatus (Radnoti, Monrovia, CA, USA) and perfused at a constant pressure of 80 mmHg with modified Krebs–Henseleit buffer (KHB) (Li et al. 2014). The temperature of the heart was maintained at 37°C by perfusion with pre-heated KHB and immersed in a water-heated glassware reservoir containing KHB. Leads for ECG recording were gently placed on the surface of the heart. The lead position was carefully adjusted to record clear ECG signals with all major waves (P, QRS and T waves). After a 20 min equilibration period, hearts were perfused with a saturate dose of ISO (10⁻⁷ M) for 15 min (Li et al. 2014). ECG was recorded continuously by a data acquisition system (Powerlab/8SP; ADI) (MacDonnell et al. 2008).

Heart weight and atrial weight measurements

Some animals were anaesthetized with sodium pentobarbital (120 mg kg⁻¹ BW, i.p.) and heparinized i.v. after the ECG or pacing procedure was performed. Then, the heart was excised. The atria and the ventricles were separated under a dissecting microscope and weighed. The weights of the heart and the atria were normalized to body weight.

SAN cell isolation

SANCs were isolated as described previously (Mangoni & Nargeot, 2001; Gao et al. 2011). Animals were anaesthetized with sodium pentobarbital (120 mg kg⁻¹ BW, i.p.) and heparinized i.v. Hearts were excised and the SAN tissue was cut out as described previously (Nilius et al. 2006; Huser et al. 2000). SANCs were isolated using a technique described by Mangoni & Nargeot (2001). For this, the heart was excised and placed into Tyrode’s solution (35°C) containing (in mM): 140 NaCl, 5.0 Heps, 5.5 Glucose, 5.4 KCl, 1.8 CaCl₂ and 1.0 MgCl₂ (pH 7.4). The SAN tissue was dissected out with a dissecting scope according to the landmarks of the heart defined by the orifice of superior vena cava, crista terminalis and atrial septum (Mangoni & Nargeot, 2001). The SAN tissue was cut into small pieces and transferred to a 10 mL test tube. Then, the tissue was rinsed with a ‘low Ca²⁺’ digestion solution containing (in mM): 140 NaCl, 5.0 Heps, 5.5 Glucose, 5.4 KCl, 0.2 CaCl₂, 0.5 MgCl₂, 1.2 KH₂PO₄, 50 Taurine and 1 mg mL⁻¹ BSA (pH 6.9). SAN tissue pieces were digested in 5 mL ‘low Ca²⁺’ solution containing collagenase type I (225 U mL⁻¹; Worthington Biochemical Corporation, Lakewood, NJ, USA), elastase (1.8 U mL⁻¹; Worthington) and protease type XIV (0.8 U mL⁻¹; Sigma, St Louis, MO, USA) for 30 min at 37°C. After digestion, the tissue was washed with 10 mL of Kraft-Bruhe medium containing (in mM): 100 potassium glutamate, 5 Heps, 20 glucose, 25 KCl, 10 potassium aspartate, 2 MgSO₄, 10 KH₂PO₄, 20 taurine, 5 creatine, 0.5 EGTA and 1 mg mL⁻¹ BSA (pH 7.2) three times and then SANCs were dissociated with a transfer pipette by mechanically stirring and pipetting the tissue chunks. Dissociated SANCs were placed at room temperature for study within 5 h.

Recording of pacemaker activity in SAN cells

The pacemaker activity of SAN cells was recorded at 35 ± 1°C in a heated chamber mounted on a Diaphot microscope (Nikon, Tokyo, Japan). The extracellular Tyrode’s solution contained (in mM): 140.0 NaCl, 5.4 KCl, 1.8 CaCl₂, 1.0 MgCl₂, 5.0 Heps, and 5.5 glucose (adjusted to pH 7.4 with NaOH). SAN cell pacemaker activity was evaluated by counting the number of beats of SANCs in segments of 1 min in duration under basal conditions and after application of ISO.

Cellular electrophysiology

Cellular electrophysiology was performed with an Axon Axopatch 2B amplifier and pClamp 9 (Molecular Devices, Sunnyvale, CA, USA). Isolated SAN cells were placed in a chamber mounted on an inverted microscope (Diaphot; Nikon). SAN cells were initially perfused with a normal physiological salt solution containing (in mmol L⁻¹): NaCl 150, KCl 5.4, CaCl₂ 1, MgCl₂ 1.2, glucose 10, sodium pyruvate 2 and Heps 5 (pH 7.4) at 37°C. Low resistance (2–4 MΩ) pipettes were filled with a solution containing (in mmol L⁻¹): Cs-aspartate 130, NMDG 10, TEA-Cl 20, Tris-ATP 2.5, Tris-GTP 0.05, MgCl₂ 1, EGTA 10 and β-escin 0.04 (pH 7.2). β-escin was used to establish a perforated-patched whole-cell
The T-type Ca channel contributes to heart rate regulation

... indicating that α-MHC promoter-driven transgene is expressed in SANCs.

Electrophysiological recording of Ca\(^{2+}\) currents in SANCs from Cav3.1 TG mice showed that total \(I_{\text{Ca}}\) and \(I_{\text{Ca-T}}\) were significantly increased in TG SANCs compared to those in WT SANCs (Fig. 1C–H). Interestingly, a decrease of \(I_{\text{Ca-L}}\) was found in TG SANCs (Fig. 1E and G), suggesting a compensatory decrease of \(I_{\text{Ca-L}}\) in TG SANCs associated with increased \(I_{\text{Ca-T}}\).

ISO increased HR more in TG but less in KO than in control mice in vivo under anaesthesia

Under conscious conditions, the sympathetic and parasympathetic nervous systems constantly regulate HRs, which disguises the differences in HR in control and Cav3.1 KO mice (Mangoni et al. 2006). Thus, we determined HR responses to ISO in anaesthetized Cav3.1 TG, Cav3.1 KO and control mice. ECGs were recorded from mice after the infusion of different concentrations of ISO via a jugular vein catheter. Although the basal HRs were similar between Cav3.1 TG, KO and control mice. ISO at low doses (10\(^{-14}\) to \(10^{-12}\) g g\(^{-1}\) BW) was able to significantly increase HRs in control mice and even more in TG mice but not in KO mice (Fig. 2A and C). ISO at doses of 10\(^{-11}\) g g\(^{-1}\) BW and higher significantly increased HR in all three groups of animals, with the greatest increases in the TG mice and the lowest increases in the KO mice. The logEC\(_{50}\) of ISO for increasing HRs in TG, control and KO mice −10.43 ± 0.16, −9.99 ± 0.12 and −9.45 ± 0.19 g g\(^{-1}\) BW, respectively (Fig. 2C). Maximal HRs after saturate ISO stimulation were highest in TG mice and lowest in the KO mice (Fig. 2). However, it appeared that the differences in maximum HR after different doses of ISO were not a result of the differences in baroreflex responses to blood pressure alterations after ISO because the systolic pressure was not different among groups at all doses of ISO (Fig. 2D). In effect, ISO did not significantly decrease systolic blood pressure at the doses of 10\(^{-16}\) g g\(^{-1}\) BW to 10\(^{-8}\) g g\(^{-1}\) BW (Fig. 2D), probably because the increase of cardiac output compensated for the effect of vascular relaxation effect of ISO. ISO slightly decreased systolic pressure at the doses of 10\(^{-7}\) and 10\(^{-6}\) g g\(^{-1}\) BW, which was no different between control, KO and TG groups of animals. These data suggest that the loss of Cav3.1 reduces, whereas overexpression of Cav3.1 sensitizes, the response of the HR to β-adrenergic stimulation.

We further determined which β-adrenergic receptor(s) was involved in this Cav3.1 mediated enhancement of HR responses to ISO. As shown in Fig. 3E–G, pretreatment of animals with ICI 118,551, a specific β2-adrenergic receptor blocker, significantly reduced HR responses to ISO stimulation at low doses (10\(^{-10}\) and 10\(^{-9}\) g g\(^{-1}\) BW) in control and TG animals but not in KO animals; at the high ISO dose of 10\(^{-8}\) g g\(^{-1}\) BW, HR increases were not changed.

Statistical analysis

Data are reported as the mean ± SEM. When appropriate, a paired or unpaired t test and one-way or two-way ANOVA with or without repeated measures was used to detect significance with Prism, version 6.0 (GraphPad, La Jolla, CA, USA). Bonferroni adjustment was performed for post hoc pairwise comparison. \(P < 0.05\) was considered statistically significant. Dose-response curves were constructed with Prism, version 6.0.

Results

T-type calcium current in mouse SAN cells is mediated by Cav3.1

To determine the identity of \(I_{\text{Ca-T}}\) in mouse SANCs, \(I_{\text{Ca-T}}\) was measured in Cav3.1 KO and wild-type control SANCs. \(I-V\) curves of total Ca\(^{2+}\) current (total \(I_{\text{Ca}}\)), \(I_{\text{Ca-L}}\) and \(I_{\text{Ca-T}}\) were constructed as shown in Fig. 1. \(I_{\text{Ca-T}}\) and \(I_{\text{Ca-L}}\) were clearly present in wild-type control SANCs but \(I_{\text{Ca-T}}\) was missing in Cav3.1 KO myocytes (Fig. 1). These data suggest that \(I_{\text{Ca-T}}\) in mouse SANCs is mediated by Cav3.1.

α-MHC promoter-driven overexpression of Cav3.1 increases \(I_{\text{Ca-T}}\) but decreases \(I_{\text{Ca-L}}\) in mouse SAN cells

To test whether α-MHC promoter–driven expression transgene was expressed in mouse SANCs, SANCs were isolated from transgenic mice carrying α-MHC promoter-driven GFP. GFP green fluorescence was clearly detected in the SANCs as shown in Fig. 1A and B,
α-MHC-GFP SANC

B α-MHC-GFP SANC

T-type and L-type Ca²⁺ currents in SA nodal cells of WT, α1G TG and α1G KO mice

C

Total I_{Ca}  
L-type I_{Ca-L}  
T-type I_{ca-T}

WT

α1G KO

α1G TG

Figure 1. Total, L- and T- type Ca²⁺ currents in SAN cells of control (WT), Cav3.1 KO and Cav3.1 TG mice. A and B, α-MHC promoter-driven expression of GFP in SANCs. A SANC from α-MHC-driven GFP transgenic mice in bright field (A) and GFP fluorescence (B). L- and T-type Ca²⁺ currents were separated by holding the cells at −90 and −50 mV. At the V_h of −50 mV, TTCC was fully inactivated and only I_{Ca-L} remained. The T-type Ca²⁺ current was obtained by subtracting the raw L-type Ca²⁺ current from the raw total Ca²⁺ current. Examples of
by ICI 118,551 pretreatment. When the animals were pre-treated with both ICI 118,551 and metoprolol, ISO did not significantly increase the HR at all ISO doses tested, which is in agreement with the addition of metoprolol to animals pretreated with ICI 118,551 and stimulated with $10^{-7} \text{g} \text{g}^{-1} \text{BW}$ ISO being seen to return the HR to baseline (Fig. 3E and F, blue line, last data point). These results suggest that, at low doses of ISO, the enhancement of chronotropic effect by Cav3.1 TTCC is preferentially mediated by $\beta 2$-AR.

ISO increased the HR of Langendorff-perfused more in Cav3.1 TG hearts but less in Cav3.1 KO hearts than control hearts ex vivo

To completely eliminate the effects of in vivo regulatory mechanisms on HRs, perfused HRs on Langendorff apparatus were used to examine intrinsic HRs at baseline and after ISO ($10^{-7}$ M, a saturated dose) stimulation ex vivo. At baseline, the HR of Cav3.1 KO hearts (396.9 ± 22.2 bpm, n = 17) was non-significantly slower than that of c57bl/6 control hearts (421.7 ± 12.4 bpm, n = 12) and TG (429.0 ± 15.4 bpm, n = 10) hearts. After the application of a saturate dose of ISO ($10^{-7}$M), the HR of Cav3.1 KO hearts (562.8 ± 23.7 bpm, n = 17) was significantly lower than that of control hearts (655.2 ± 17.0 bpm, n = 12, P < 0.05) and TG hearts (723.6 ± 18.4 bpm, P < 0.05) (Fig. 3A and B). The percentage of increase in HR was significantly less in the KO mice (28.1 ± 3.4 %, n = 12) than in the control mice (45.8 ± 3.2%, n = 9, P < 0.05) and TG mice (73.8 ± 8.1%) (Fig. 3C). These data were consistent with the results obtained from in vivo hearts.

Because TTCC is also expressed in AV node and plays an important role in impulse conduction, we analysed PR intervals of Cav3.1 TG and KO and control hearts before and after the application of ISO. We found that the loss of Cav3.1 prolonged the PR interval in KO hearts (control versus KO: 32.1 ± 0.3 ms, n = 12 versus 34.9 ± 0.4 ms, n = 17, P < 0.05), whereas Cav3.1 overexpression (28.4 ± 0.6 ms, n = 10) shortens the PR interval (Fig. 3D). ISO shortened PR intervals in all Langendorff perfused hearts but to a greater extent in TG hearts and to a less extent in KO hearts compared to control hearts (Fig. 3E). To rule out the possibility that the prolonged PR interval was a result of changes in the conduction of the atria, we paced the atria via an intra-RA catheter and measured the time from the end of the pacing until the start of the QRS complex in the surface ECG in vivo. In agreement with the results of the study by Langendorff perfused heart study (Figure 2), the time from the end of atria pacing till the start of the QRS was prolonged in the KO animals but shortened in the TG animals compared to the control (Fig. 3F and G), implicating changes of intrinsic properties of the SA node and/or Purkinje fibres. The atria or the ventricles were not enlarged/hypertrophied in either Cav3.1 TG or KO animals (Fig. 3H), as reported previously (Jaleel et al. 2008). Nakayama et al. 2009). These data suggest that Cav3.1 plays important roles in regulating both SAN and AVN at baseline and after $\beta$-adrenergic stimulation.

Cav3.1 expression affects sinoatrial nodal cell automaticity and its regulation by $\beta$-adrenergic stimulation

We next examined the beating rates of SANCs from Cav3.1 KO, TG and control mice. Basal beating rates of Cav3.1 KO SANCs were significantly slower than those of TG and control SANCs. However, basal beating rates of TG SANCs were no different from those of control SANCs (Fig. 4A).

To determine the effects of different doses of ISO on the beating rate of SANCs, three concentrations of ISO ($10^{-9}$, $10^{-8}$ and $10^{-7}$M) were applied to SANCs. There were dose-dependent increases of beating rates of all groups of SANCs. ISO at the doses of $10^{-8}$ and $10^{-7}$M increased spontaneous beating rate to a lesser extent in KO SANCs but to a greater extent in TG SANCs (Fig. 4A). The percentage of the increase of HR by $10^{-7} \text{M}$ ISO versus baseline was the greatest in TG SANCs (41.5 ± 2.5%), greater in control SANCs (31.14 ± 1.7%) and the least in KO SANCs (24.9 ± 1.8%, P < 0.05).

$\text{ICa-T}$ in SANCs is increased by ISO

The data obtained in the present study suggest that Cav3.1 participates in HR regulation by $\beta$-adrenergic stimulation. We then tested whether $\text{ICa-T}$ can be regulated directly by $\beta$-adrenergic agonist in SANCs. The ISO effect on $\text{ICa-T}$ was monitored by recording $\text{ICa}$ at $-30 \text{mV}$ depolarized from $-90 \text{mV}$ every 20 s. Figure 5 shows a significant upregulation of $\text{ICa-T}$ mediated by Cav3.1 by ISO in both WT and TG SANCs.
**Figure 2. ISO dose–HR responses in sedated Cav3.1 KO, Cav3.1 TG and control mice**

Mice were anaesthetized and a catheter was inserted into the left jugular vein to infuse a series of ISO from $10^{-16}$ g g$^{-1}$ BW to $10^{-6}$ g g$^{-1}$ BW. A, examples of HR changes in response to different doses of ISO in anaesthetized Cav3.1 KO (blue) and TG (red) and control (black) mice. B, examples of ECG recorded at baseline and at maximal HR after ISO. C, average HR at different concentrations of ISO in Cav3.1 KO, TG, and control mice. HR–ISO dose relationship were fit with a dose–response curve to derive EC$_{50}$. The inserted table shows the logEC$_{50}$ and maximal percentage of HR increase in TG, KO and control mice. @ $P < 0.05$, @@ $P < 0.01$ and @@@ $P < 0.001$ TG versus control; # $P < 0.05$, KO versus control; $^\dagger$ $P < 0.001$ TG versus control; $^\ddagger$ $P < 0.001$, KO versus control; $^\S$ $P < 0.05$, $^\SS$ $P < 0.001$ and $^\SSS$ $P < 0.0001$, TG versus KO at the same ISO dose determined by two-way repeated ANOVA and post hoc tests with Bonferroni adjustment. D, left ventricular systolic pressure at different doses of ISO. E–G, maximum HRs at different i.p. doses of ISO ($10^{-10}$, $10^{-9}$ and $10^{-8}$ g g$^{-1}$ BW) without or with ICI 118,551 (2 μg g$^{-1}$ BW, i.p., 5 min) or with ICI 118,551 + metoprolol (2 μg g$^{-1}$ BW, i.p., 5 min) in sedated control, KO and TG animals. $^\S$ $P < 0.05$, without pretreatment versus with ICI pretreatment; $^\dagger$ $P < 0.05$, without treatment versus with ICI + metoprolol pretreatment; $^\S$ $P < 0.05$, pretreatment with ICI versus pretreatment with ICI + metoprolol. At the same ISO dose, significance was determined by two-way repeated ANOVA and post hoc tests with Bonferroni adjustment. [Colour figure can be viewed at wileyonlinelibrary.com]
Discussion

Previously, we reported that β-adrenergic stimulation could increase Cav3.1 TTCC activity in mouse ventricular myocytes overexpressing Cav3.1 via a PKA-dependent mechanism (Li et al. 2012). The present study further explored whether this upregulation of TTCC activity by β-adrenergic stimulation contributed to the HR increase and AV conduction acceleration by the SAS. We found that: (i) Cav3.1 mediates I_{Ca-T} in SANCs because Cav3.1 KO eliminated I_{Ca-T} but Cav3.1 overexpression augmented I_{Ca-T} in SANCs; (ii) Cav3.1 KO reduced the sensitivity and extent of HR upregulation and AV conduction acceleration by β-adrenergic stimulation in vivo and ex vivo, whereas Cav3.1 overexpression had the opposite effects; (iii) Cav3.1 KO reduced spontaneous beating rates and its response to ISO, whereas Cav3.1 TG had contrasting effects; and (iv) I_{Ca-T} in wild-type and Cav3.1 TG SANCs was upregulated by ISO.

The role of Cav3.1 in basal HR generation

Our electrophysiological studies showed enhanced I_{Ca-T} in SANCs of Cav3.1 transgenic (driven by α-MHC promoter) mice and an absence of I_{Ca-T} in SANCs of Cav3.1 KO mice (Fig. 1). Ex vivo KO hearts or in vitro KO SANCs showed slower beating rates than those of control hearts and SANCs, suggesting an important role of Cav3.1 in HR generation. By contrast, ex vivo Cav3.1 transgenic hearts and in vitro Cav3.1 TG SANCs did not have higher beating rates than control hearts and SANCs. This could be because the role of Cav3.1 in TG mice was masked by the compensatory changes of other channels and Ca^{2+} handling proteins in the TG SANCs. The results of the present study show that the L-type Ca^{2+} current is reduced in the Cav3.1 TG SANCs (Fig. 1). Our previous study of Cav3.1 TG hearts showed changes of several Ca^{2+} handling proteins in the ventricles including decreased L-type Ca^{2+} channel, increased SERCA2a and reduced phospholamban expression (Jaleel et al. 2008). Because L-type Ca^{2+} current and spontaneous cyclic Ca^{2+} release are important for SANC automaticity, these changes could account for the observation that the basal HR in Cav3.1 TG mice is not increased. Therefore, these data from Cav3.1 TG mice do not undermine the suggestion that Cav3.1 plays a role in HR generation (Huc et al. 2009).

The role of Cav3.1 in HR regulation by β-adrenergic stimulation

The focus of the present study was to examine the role of I_{Ca-T} stimulated by β-adrenergic in HR regulation by the β-adrenergic system. We showed that I_{Ca-T} in mouse SANCs can be increased by β-adrenergic stimulation (Fig. 5). When the mice were anaesthetized, the sensitivity of the HR to ISO stimulation was TG > control > KO (Fig. 2). The beating rates of Cav3.1 KO hearts and SANCs were less sensitive to ISO, whereas these rates of Cav3.1 TG hearts and SANCs were more sensitive to ISO (Figs 3–5). Collectively, these data suggest the upregulation I_{Ca-T} in SANCs contributes to HR upregulation by β-adrenergic stimulation. Our data further suggest that this is
Langendorff-perfused hearts

A, examples of ECG recorded at baseline and at maximal HR after ISO in isolated and perfused Cav3.1 KO (blue) and TG (red) and control (black) mice. B, HR before and after the application of 10^{-7} M ISO. C, the percent increase of HR by ISO in Cav3.1 KO and TG, and control mice. D, PR intervals of Langendorff-perfused control (black), Cav3.1 KO (blue) and TG (red) hearts before and after the application of ISO. E, the percentage of PR interval decrease (100 – PR interval after ISO/baseline PR interval × 100) induced by ISO in c57bl/6 control and Cav3.1 KO mice.

Figure 3. HR and PR intervals of Langendorff perfused hearts from Cav3.1 KO, TG and control WT mice before and after ISO (10^{-7} m)

A, examples of ECG recorded at baseline and at maximal HR after ISO in isolated and perfused Cav3.1 KO (blue) and TG (red) and control (black) mice. B, HR before and after the application of 10^{-7} M ISO. C, the percent increase of HR by ISO in Cav3.1 KO and TG, and control mice. D, PR intervals of Langendorff-perfused control (black), Cav3.1 KO (blue) and TG (red) hearts before and after the application of ISO. E, the percentage of PR interval decrease (100 – PR interval after ISO/baseline PR interval × 100) induced by ISO in c57bl/6 control and Cav3.1 KO mice. F, examples of surface ECG and atrial electrogram before and during atrial pacing at 600 Hz. G, PR intervals during atrial pacing-induced ventricular beats. H, heart weight or atrial weight to body weight ratios of control, KO and TG animals. *P < 0.05, **P < 0.01 and ***P < 0.001. Data in (B) and (D) were analysed by two-way repeated ANOVA and post hoc tests with Bonferroni adjustment; data in (C) and (E) were analysed by one-way ANOVA and post hoc tests with Bonferroni adjustment. n, number of animals studied. [Colour figure can be viewed at wileyonlinelibrary.com]
preferentially mediated by β2-AR, especially at low level of β-adrenergic stimulation. We suspect that the Cav3.1 TTCC could be located close to or preferentially coupled to β2-AR signalosome.

In recent years, it has been proposed that β-adrenergic regulation of HR is mediated not only by the direct effect of cAMP on funny channels, but also by cAMP/PKA effect on SANC Ca^{2+} handling (Lakatta et al. 2010; Lau et al. 2011). Therefore, it is possible that β-adrenergic/PKA regulation of Cav3.1 participates in the positive chronotropic effects of β-adrenergic agonists by enhancing both phase 4 diastolic depolarization current (‘membrane clock’) and SANC Ca^{2+} handling (‘calcium clock’). It appears that the compensatory decrease of I_{Ca-L} in Cav3.1 TG SANCs does not impair the capability of increasing Cav3.1 by β-adrenergic stimulation to increase HR. Thus, our data suggest that the expression level of I_{Ca-T} is a direct modulator the sensitivity of the HR response to
β-adrenergic stimulation. Because Cav3.1 also mediates $I_{Ca-T}$ in human SANCs (Chandler et al. 2009) and Cav3.1 is highly homologous between human and mouse (Monteil et al. 2000), this mechanism probably also participates in human HR regulation.

The role of Cav3.1 in conduction through the AVN

It is well known that sympathetic/β-adrenergic stimulation on the heart increases the conduction rate of the AV node and thus shortens the PR interval. However, the exact mechanism of this acceleration by sympathetic/β-adrenergic stimulation is still not entirely clear. Because HCN4 (mediating $I_f$) is expressed in AVN myocytes, cAMP increase after sympathetic/β-adrenergic stimulation may enhance $I_f$ and thus enhance AVN conduction (Mesirca et al. 2015). In addition, Kim et al. (2010) showed that ISO stimulation of rabbit AVN elicited a late diastolic Ca$^{2+}$ elevation that could enhance diastolic depolarization via sodium/calcium exchange. Cav3.1 plays a role in AV nodal conduction (Mangoni et al. 2006), although its role in AVN conduction regulation by the SAS has not be reported. We found that Cav3.1 expression positively affects AVN conduction regulation by ISO (Fig. 3 D and E). As in the SAN cells, enhanced Cav3.1 activity by ISO could contribute to the AVN conduction acceleration probably by providing more late phase 4 depolarizing current directly (‘current clock’) and by enhancing late diastolic Ca$^{2+}$ elevation (‘Ca$^{2+}$ clock’). However, these mechanisms may need to be studied in large mammal preparations because the isolation of AVN cells from mice can be challenging. Alternatively, optical mapping of AVN can be used to determine whether the AVN conduction or the Purkinje fibre conduction is slowed, although our pacing study ruled out atrial change for the PR interval prolongation.

Significance and limitations

The findings of the present study add a new contribution to the regulation of HRs by the SAS system, which is critical for normal cardiac function. In addition, it is possible that altered regulation of the TTCC by the SAS could cause cardiac dysrhythmias such as tachycardia, bradycardia and AV conduction block. Furthermore, inhibiting TTCC upregulation by the SAS could be adopted as a way of slowing down the HR, such as in heart failure treatment.

We caution that murine cardiac physiology is different from that of human and other large mammals, especially in terms of the autonomic control of HR (Kaese & Verheule, 2012). The HR in mice is much faster than that in humans. Accordingly, the shape and duration of action potentials, the underlying ionic currents and Ca$^{2+}$ handling in mice all support heart function at such fast HRs. Although the conduction of action potential through the AV node is similar between human and mice, conduction through the atria and ventricles is different between these two species. As such, we need to be conservative when extrapolating our results to humans and other large mammals.

Furthermore, in the present study, we used ISO (a non-selective β-adrenergic agonist activating all three β-ARs) to mimic the effect of sympathetic stimulation of the sinoatrial node and AVN. Previous studies have shown that sympathetic stimulation and ISO (or other exogenous β-AR agonists) stimulation often do not produce the same effect on HR and conduction rate acceleration (Du et al. 1996; Mantravadi et al. 2007). Sympathetic nerves release norepinephrine that is different from ISO

Figure 4. ISO dose–response relationships of spontaneous beating rates of SAN cells of Cav3.1 TG, KO and control mice

A, spontaneous beating rates of control, Cav3.1 KO and TG SANs before and after the application ISO (10$^{-9}$ to 10$^{-7}$). B, percentage of increases of SAN beating rates by 10$^{-7}$ ISO in control; Cav3.1 KO and TG SANCs. ‘n’ is the number of cells from three animals from each group. $^\#P < 0.05$ and $^\#\#P < 0.01$, TG versus control; $^\#P < 0.05$ and $^\#\#P < 0.01$, KO versus control; $^\#P < 0.05$, $^\#\#P < 0.01$ and $^\#\#\#P < 0.001$, TG versus KO at the same ISO dose determined by two-way repeated ANOVA and post hoc tests with Bonferroni adjustment. $B: ^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, analysed by one-way ANOVA and post hoc tests with Bonferroni adjustment. [Colour figure can be viewed at wileyonlinelibrary.com]
The T-type Ca channel contributes to heart rate regulation and the distribution of nerve ends and post-synaptic adrenergic receptors, as well as ion channels, affects the effect of sympathetic stimulation in specific ways. Accordingly, further studies confirming that the Cav3.1 T-type Ca\(^{2+}\) channel plays an important role in the sympathetic regulation of HR and conduction need to be performed by direct stimulation of the sympathetic nerves.

**Figure 5.** \(I_{\text{Ca-T}}\) in SANCs from control and Cav3.1 TG mice is increased by ISO (1 \(\mu\)M)

A and B, \(I_{\text{Ca-T}}\) traces recorded in one control and one TG SANC bathed in nifedipine (10 \(\mu\)M) then nifedipine (10 \(\mu\)M) + ISO (1 \(\mu\)M). The recording was every 20 s but, for clarity, only \(I_{\text{Ca-T}}\) traces every 40 s (A) or 80 s (B) are shown. The insert is the voltage clamp protocol. C and D, the time course of ISO [nifedipine (10 \(\mu\)M) + ISO (1 \(\mu\)M)] effect on \(I_{\text{Ca-T}}\) amplitude recorded at −30 mV depolarized from −90 mV in the presence of nifedipine (10 \(\mu\)M). E and F, the percentage of increases of \(I_{\text{Ca-T}}\) in WT and Cav3.1 TG SAN cells. A paired t test was used for statistics.
Conclusions

Our data collectively suggest that Cav3.1 activity enhancement by β-adrenergic stimulation plays an important role in HR regulation by the SAS in mice. Abnormal Cav3.1 regulation by the SAS system may contribute to abnormal cardiac automaticity. The TTCC can be a target for potential HR slowing treatments.

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Additional information

Competing interests

The authors declare that they have no competing interests.

Author contributions

YXL, XXZ, CZ, XYZ, YL and ZQ performed the study, analysed the data and drafted the manuscript. CS and MXT handled the animals. YZP, JDM and SRH criticized and revised the manuscript. MXX and XWC organized the study, analysed some of the data, drafted and revised the manuscript. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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