Bradycardia and Slowing of the Atrioventricular Conduction in Mice Lacking CaV3.1/α1G T-Type Calcium Channels

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Abstract—The generation of the mammalian heartbeat is a complex and vital function requiring multiple and coordinated ionic channel activities. The functional role of low-voltage activated (LVA) T-type calcium channels in the pacemaker activity of the sinoatrial node (SAN) is, to date, unresolved. Here we show that disruption of the gene coding for Ca,3.1/α1G T-type calcium channels (caca1g) abolishes T-type calcium current (I_{CaL}) in isolated cells from the SAN and the atrioventricular node without affecting the L-type Ca^{2+} current (I_{CaL}). By using telemetric electrocardiograms on unrestrained mice and intracardiac recordings, we find that caca1g inactivation causes bradycardia and delays atrioventricular conduction without affecting the excitability of the right atrium. Consistently, no I_{CaL} was detected in right atrium myocytes in both wild-type and Ca,3.1/^- mice. Furthermore, inactivation of caca1g significantly slowed the intrinsic in vivo heart rate, prolonged the SAN recovery time, and slowed pacemaker activity of individual SAN cells through a reduction of the slope of the diastolic depolarization. Our results demonstrate that Ca,3.1/T-type Ca^{2+} channels contribute to SAN pacemaker activity and atrioventricular conduction. (Circ Res. 2006;98:1422-1430.)

Key Words: pacemaker activity | T-type calcium channel | sinoatrial node | conduction | knockout mice

The initiation of the heartbeat requires coordination between the automaticity of the sinoatrial node (SAN) and excitability of the atrioventricular (AV) conduction tissue: the AV node (AVN) and the His–Purkinje fiber network. SAN automaticity is caused by the presence of the diastolic depolarization.1 Multiple classes of ionic channels are expressed in the SAN,1,2 but the precise mechanism initiating the diastolic depolarization has not been entirely elucidated, and the relative contribution of different ionic channels in establishing the heart rate under specific physiological conditions is still a matter of debate.3–5 Strong functional, pharmacological, and genetic evidence show that the hyperpolarization-activated currents I_{K1}6,7 and I_{CaL}8–10 play major roles in controlling the diastolic depolarization after decaying of the fast component of delayed-rectifier K+ currents (I_{Kp}). Diastolic release of Ca^{2+} mediated by ryabodine receptors (RyRs) has been indicated as an important mechanism for controlling SAN pacemaking under activation of the β-adrenergic receptor.11 The SAN also expresses neuronal tetrodotoxin (TTX)-sensitive and cardiac TTX-resistant voltage-dependent Na+ currents (I_{Na}).12 TTX-sensitive I_{Na} is involved in SAN pacemaking in the newborn rabbit13 and in the adult mouse.12,14 The cardiac TTX-resistant SCN5A-mediated I_{Na} is important for conduction from the SAN to the atrium and in intranodal conduction.15 I_{Na} is also expressed in the rabbit16 and guinea pig17 AVN and contributes to the fast AV conduction pathway.18 Consistently, heterozygous mice lacking SCN5A channels have major AV conduction dysfunction.19

I_{CaL} has also been proposed to contribute to pacemaking in primary SAN20 as well as in latent pacemaker cells of the right atrium (RA).21 I_{CaL} is expressed in pacemaker cells of mammals, including the rabbit20 and the mouse.22 I_{CaL} is also expressed in the cardiac conduction system, the rabbit AVN,18 and in canine Purkinje cells.23,24 The functional role of I_{CaL} in the cardiac primary pacemaker and conduction tissue remains to be elucidated.

Molecular cloning of 3 T-type Ca^{2+} channel pore-forming subunits, namely the CaV3.1/α1G, CaV3.2/α1H, and CaV3.3/α1I subunits, respectively, in the SAN of mice lacking caca1g by using reverse transcriptase polymerase chain reaction.

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subunits, has fostered investigation of the role of native $I_{\text{Ca,T}}$.25
Here, we describe that CaV3.1 knockout (CaV3.1$^{-/-}$) mice lack $I_{\text{Ca,T}}$ in both SAN pacemaker and AVN cells and display slowed pacemaker activity and AV conduction. Our results constitute the first direct functional demonstration of the participation of Cav3.1 channels in pacemaking and cardiac conduction.

Materials and Methods
The generation of the CaV3.1$^{-/-}$ mouse line was originally described by Kim et al.26 The experimental procedure for electrophysiological recording of isolated cells from the SAN, the AVN, and the RA is described in the expanded Materials and Methods section in the online data supplement, available at http://circres.ahajournals.org. Detailed procedures for in vivo surface and intracardiac electrocardiograms (ECGs), measurement of blood pressure, RT-PCR, and quantitative RT-PCR experiments (supplemental Figure II) and numerical modeling of pacemaker activity are available in the expanded online Materials and Methods section. Results are presented as the mean±SEM. Statistical significance was assessed by the unpaired Student’s $t$ test. A value of $P<0.05$ was considered as statistically significant. N indicates the number of mice used and n the number of cells considered.

Results
CaV3.1$^{-/-}$ SAN Pacemaker Cells Lack $I_{\text{Ca,T}}$
Electrophysiological recordings obtained on SAN cells of wild-type (WT) and CaV3.1$^{-/-}$ animals revealed that $I_{\text{Ca,T}}$ related to CaV3.1 is a major component of the total Ca$^{2+}$ current ($I_{\text{Ca}}$) in SAN cells (Figure 1). Figure 1A shows representative $I_{\text{Ca}}$ traces recorded from a holding potential (HP) of $-90$ mV in WT (Figure 1Aa) and CaV3.1$^{-/-}$ (Figure 1Ab) SAN cells. No difference in the cell capacitance was detected (see supplementary Figure I). Corresponding current–voltage ($I$-$V$) curves are presented in Figure 1B (filled symbols). $I_{\text{Ca,L}}$ was measured by applying depolarizing steps from a HP of $-55$ mV (Figure 1B, open symbols), revealing $I_{\text{Ca,T}}$ by subtraction of records from a HP of $-90$ mV from those obtained from a HP of $-55$ mV (Figure 1Ba, dotted line). Consistently, switching the HP from $-90$ to $-55$ mV significantly shifted $I_{\text{Ca}}$ voltage for half activation ($V_{0.5\text{act}}$) to more positive values ($-45±1$ mV; $k=5.7±0.6$ mV from a HP of $-90$ mV and $-28±1$ mV; $k=6.3±0.3$ mV from a HP of $-55$ mV, $N=9$, n=17, $P<0.05$) in all tested cells (n=17), revealing further a $V_{0.5\text{act}}$ of $-47±1$ mV for the net $I_{\text{Ca,T}}$ (Figure 1Ba, dotted line). In contrast to WT SAN cells, none
of the tested Ca,3.1−/− cells displayed detectable I_{Ca,T} (Figure 1Bb). Indeed, in Ca,3.1−/− SAN cells, switching to a HP of −55 mV did not alter significantly voltage dependence of I_{Ca} activation (HP, −90 mV: V_{0.5act} = −30±1 mV; k = 8.0±1.3 mV, n = 14; and HP −55 mV: −28±1 mV; k = 6.3±0.3 mV, N = 10, n = 15, P = NS). Subtraction between traces from HP −90 mV and from HP −55 mV identified no residual I_{Ca,T}, because current waveforms displayed slow inactivation kinetics (Figure 1Bb, inset) and no criss-crossing of the current traces. A comparison of the current density values in WT and Ca,3.1−/− SAN cells at HP −90 mV is shown in Figure 1C (black and white bars, respectively) to estimate the I_{Ca,T} component related to Ca,3.1. At −40 mV, I_{Ca,T} density was 6.8±1.6 pA/pF (+/+), N = 9, n = 18 and I_{Ca,L} density was 2.6±0.7 pA/pF, (−/−), N = 10, n = 14. I_{Ca,L} density was not significantly affected by inactivation of Ca,3.1 channels (Figure 1D). Indeed, I_{Ca,L} peak densities at −10 mV (HP, −55 mV) were 6.0±1.6 pA/pF, N = 9, n = 15; and 4.5±0.6 pA/pF, N = 10, n = 11 in Ca,3.1−/− and WT SAN cells, respectively. Taken together, these data showed that inactivation of Ca,3.1 channels abolished I_{Ca,T} and had no significant effect on I_{Ca,L} in SAN cells.

To determine whether the pharmacological sensitivity of the SAN I_{Ca,T} is consistent with the expression of the Ca,3.1 subunit, we tested its sensitivity to Ni^{2+} ions (Figure 2A and 2B). Application of Ni^{2+} concentrations of 50 and 200 μmol/L inhibited 26±9% (N = 2, n = 4) and 61±10% (n = 9) of I_{Ca,T}, respectively. Because the IC_{50} values for Ni^{2+} on recombinant Ca,3.1 and Ca,3.2 T-type channels are in the range of 100 to 200 μmol/L and 5 to 10 μmol/L, respectively, these results indicate that I_{Ca,T} in mouse adult SAN cells is generated by the Ca,3.1 subunit. Consistent with these findings, real-time RT-PCR experiments showed that the Ca,3.1 subunit is the predominant T-type Ca^{2+} channel isotype in the SAN of the adult mouse (supplemental Figure I). Furthermore, application of 2 μmol/L of the 1,4-dihydropyridine I_{Ca,L} blocker isradipine on Ca,3.1−/− SAN cells blocked I_{Ca,L} by 93±4% (N = 5, n = 7, Figure 2C and 2E) from a HP of −90 mV and by 100% from a HP of −55 mV (N = 5, n = 8, Figure 2D and 2F). Such a sensitivity to isradipine further confirmed that the remaining I_{Ca} in SAN cells from Ca,3.1−/− mice was I_{Ca,L}.

**Figure 2.** A and B, Sample traces showing partial block of I_{Ca,T} by 200 μmol/L Ni^{2+} in WT SAN cells (A) and histogram of the percentage of inhibition at 50 and 200 μmol/L Ni^{2+} (B). C and D, Isradipine (Isr) block of I_{Ca} in Ca,3.1−/− SAN cells measured from a HP of −90 mV (C) and −55 mV (D) as indicated. Note that no residual dihydropyridine-resistant I_{Ca,T} is recorded in cells lacking Ca,3.1 channels at the corresponding peak of I_{Ca,L} recorded in WT cells (−35 mV). E and F, Corresponding averaged I-V curves in control conditions (filled boxes) and 2 μmol/L isradipine (open boxes).

**Sedated Ca,3.1−/− Mice Have Intrinsic Slower Heart Rate and Prolonged AV Conduction**

We next performed surface ECG recordings on sedated WT and Ca,3.1−/− mice (Figure 3). Because the heart rate is highly regulated by the balance between the sympathetic and parasympathetic inputs, which depends on the levels of stress and anesthesia, ECGs were also recorded after injection of propranolol and atropine to block the autonomic nervous system (ANS).28 Recording examples collected from WT (+/+ and Ca,3.1−/− (−/−) mice before and after the ANS block are presented in Figure 3A. The loss of the Ca,3.1 subunit induced a significant (9%) prolongation of the atrioventricular conduction (PQ interval) under baseline conditions (34±1
The spontaneous activity of isolated SAN cells was studied to evaluate whether the slowing of the intrinsic heart rate observed in vivo in CaV3.1−/− mice was associated with dysfunction of SAN cell automaticity. Representative recordings obtained on WT and CaV3.1−/− SAN cells are presented in Figure 4A (upper and lower traces, respectively). Analysis of these recordings showed that inactivation of cacna1g gene resulted in a 37% slowing of the cellular beating rate (163±13 bpm, N=4, n=15 in CaV3.1−/−, and 234±19 bpm, N=5, n=14 in SAN cells from WT mice; P<0.01; Figure 4B). Slowing of cellular pacemaking was accompanied by a reduction of the diastolic depolarization slope of −44±% (Figure 4C). No significant changes in the maximum diastolic potential (MDP) and the voltage threshold of the action potential upstroke (Ep) were observed (MDP: −56.9±1.3 mV in WT, n=15, and −58.2±1.4 mV for CaV3.1−/− SAN cells, n=14; Ep: −45±1.3 mV in WT and −43±1.4 mV in CaV3.1−/− SAN cells; Figure 4D). Also, the action potential duration (147±15 and 141±10 ms for CaV3.1−/− and WT SAN cells, respectively; Figure 4E) and the action potential amplitude (APA) (92±8 and 90±8 mV for CaV3.1−/− and WT SAN cells, respectively; not shown) were comparable in SAN cells from WT and CaV3.1−/− mice.

**Intracardiac Electrophysiology Study of CaV3.1−/− Mice**

The AV conduction delay in mice lacking CaV3.1 channels was measured under baseline intracardiac recording conditions. Eight of 10 WT and 7 of 9 CaV3.1−/− mice had a distinctly visible His-bundle electrogram (Figure 5A). In this group of animals, atrial-His (AH) conduction times were significantly longer in CaV3.1−/− mice (31±1 ms) than in WT animals (25±1 ms; P<0.001; Figure 5B). However, His-ventricular (HV) intervals were comparable (11±1 and 10±1 ms for WT and CaV3.1−/− mice, respectively; P>0.05). Effective refractory periods at the atrial (AERP), atrioventricular (AVERP), and ventricular (VERP) levels were investigated at a basic cycle length of 100 ms (Figure 5C). AERP and VERP were not significantly different between WT and CaV3.1−/− mice. In contrast, the AVERP was significantly longer in CaV3.1−/− mice (58±6 ms; N=8) than in WT animals (44±2 ms; N=10; P<0.05). Decremental atrial pacing revealed that Wenckebach cycle length was significantly longer in CaV3.1−/− mice than in WT mice (85±5 ms and 67±2 ms, respectively; P<0.01; Figure 5C). Atrial pacing confirmed SAN dysfunction. Indeed, the corrected SAN recovery time (cSNRT) was longer in CaV3.1−/− mice than in WT mice (31±5 in CaV3.1−/− and 17±2 ms in WT mice P<0.05; n=7 and 9, respectively).

**AVN Cells From CaV3.1−/− Mice Lack ICaT**

We then tested whether dysfunction of AV conduction observed on inactivation of CaV3.1 channels was associated with downregulation of I_{CaT} in the AVN. I_{CaT} and I_{CaL} were recorded in isolated AVN cells using the same recording protocols as for SAN cells (Figure 6). I_{CaT} was found in all the WT AVN cells investigated (Figure 6A). Indeed, switching the HP from −90 to −60 mV significantly shifted the V_{0.5act} of the total I_{Ca} from −34±0.7 mV to −23±0.8 mV (n=7, P<0.05). In contrast, no I_{CaT} could be recorded in CaV3.1−/− AVN cells (Figure 6Ab), and switching the HP from −90 to −60 mV did not shift the V_{0.5act} value (−25±1.3 mV to

**SAN Cells From CaV3.1−/− Mice Display Slowed Pacemaker Activity**

The spontaneous activity of isolated SAN cells was studied to evaluate whether the slowing of the intrinsic heart rate observed in vivo in CaV3.1−/− mice was associated with

ms, N=11, and 37±1 ms, N=12 for WT and CaV3.1−/− mice, respectively; P<0.05). The heart beat (RR interval), P wave duration, and QRS interval (Figure 3B), as well as the QTc interval (data not shown; 62±1 ms and 61±1 ms for WT and CaV3.1−/− mice, respectively; P>0.05), were not significantly modified. In conditions of ANS block, the intrinsic heart rate was significantly slowed by −10%. Indeed, the RR interval was significantly longer in CaV3.1−/− mice than in WT animals (144±2 ms and 132±2 ms, respectively; P<0.05; Figure 3B). The PQ interval was also markedly prolonged (34±1 ms and 40±1 ms in WT and CaV3.1−/− animals, respectively; P<0.001; Figure 4B). The other ECG parameters were not significantly modified.
compensation for the lack of activation of other ionic channels involved in SAN automaticity can 
be compensated (40 seconds) was unchanged (Table), suggesting that activation of CaV3.1 
maximum cardiac frequency during short periods of activity (Figure 7B and the Table). The 
altered in CaV3.1 
the degree of autonomic regulation of heart rate was not 
not significantly modified between WT and CaV3.1 
Table). The mean heart rate was significantly slowed in 
freely moving CaV3.1 
both the diastolic depolarization in the SAN and impulse 
Telemetric recordings also confirmed the prolongation of the PQ interval in Cav3.1 
mination (SDD) (C) SAN cells. D, Box histograms showing the average MDP and the 
E th in SAN cells from WT (+/+) (open circles) and Cav3.1 
Corresponding 25th and 75th percentile values are shown in phase in the right panel. B and 
Box and C, Histograms of the average bpm value (B) and the slope of the diastolic depolarization (SDD) (C) SAN cells. D, Box histograms showing the average MDP and the 
E th in SAN cells from WT (+/+) (open circles) and Cav3.1 
Corresponding 25th and 75th percentile values are shown in phase in the right panel. B and 

Bradycardia in Conscious Unrestrained CaV3.1 
Both the heart rate and the AV conduction were altered in 
freely moving CaV3.1 
when considering a 24-hour period or during the day and night periods (Figure 7B and the Table). The maximum cardiac frequency during short periods of activity (40 seconds) was unchanged (Table), suggesting that activation of other ionic channels involved in SAN automaticity can compensate for the lack of I Ca,t during strong activation of the sympathetic nervous system. Spectral analysis of RR signals in the frequency domain revealed no differences between the 2 groups of mice. Total spectral power, ultralow (ULF), very-low (VLF), low (LF), and high (HF) frequencies were not significantly modified between WT and CaV3.1 
Figure 7C, data not shown for ULF and VLF), indicating that the degree of autonomic regulation of heart rate was not altered in CaV3.1 

Discussion 
The major finding of this study is that genetic inactivation of the 
CaV3.1/α1G T-type Ca2+ channels in mice results in a significant slowing of the heart rate and AV conduction. Downregulation of I Ca,t in both SAN and AVN cells of 
the lack of CaV3.1 channels in isolated SAN cells induces slowing of pacemaker activity through a reduction of the slope of the diastolic depolarization. Our study provides novel and compelling genetic evidence for a direct contribution of CaV3.1 channels in the setting of the mammalian cardiac impulse generation and propagation by contributing to both the diastolic depolarization in the SAN and impulse conduction through the AVN.
Cav3.1 channels did not significantly modify the expression of Cav3.1 subunits do not compensate for the lack of Cav3.1 channels in observed on recombinant Cav3.1-mediated 

First, bradycardia was evident in sedated mice under pharmacological blockade of the ANS input for heart rates that

Figure 5. Cardiac electrophysiology studies in anesthetized WT (+/+ ) and Cav3.1−/− (−/−) mice. A, Representative surface lead I ECG and intracardiac (intra.) recordings under baseline conditions. B, Histograms of the average AH and HV intervals measured in baseline conditions in WT (open bars; N=8) and Cav3.1−/− (filled bars; N=7) mice. RR intervals were 111±3 and 122±4 ms for WT and Cav3.1−/− mice, respectively (P<0.05). C, Histograms of the average atrial effective refractory period (AERP), atrioventricular ERP (AVERP), ventricular ERP (VERP), and AV Wenckebach cycle length (Wenck.) in WT (open bars; N=9 to 10) and Cav3.1−/− (filled bars; N=6 to 8) mice.

I_{Ca,T} in SAN and AVN Cells Following Inactivation of Cav3.1 Channels

A striking observation of this study is that I_{Ca,T} in the mouse SAN is predominantly generated by the Cav3.1 subunit because we found no evidence for a residual I_{Ca,T} in Cav3.1−/− mice in both SAN (Figure 1) and AVN cells (Figure 6). The low sensitivity of SAN I_{Ca,T} to Ni²⁺ is consistent with that observed on recombinant Cav3.1-mediated I_{Ca,T}.27 Also consistent with our general findings is the recent observation that Cav3.2−/− mice showed no ECG alterations.29 Inactivation of Cav3.1 channels did not significantly modify the expression of I_{Ca,L} in Cav3.1−/− in both SAN and AVN cells. Our data show, therefore, that expression of Cav3.2, Cav1.3, and Cav1.2 subunits do not compensate for the lack of Cav3.1 channels in Cav3.1−/− mice.

Bradycardia and Atrioventricular Dysfunction in Cav3.1−/− Mice

ECGs and intracardiac recordings document both bradycardia and slowing of the atrioventricular conduction in Cav3.1−/− mice. Several lines of evidence indicate that bradycardia is attributable to slowing of the pacemaker activity in the SAN. First, bradycardia was evident in sedated mice under pharmacological blockade of the ANS input for heart rates that

were comparable with the mean heart rate observed in freely moving mice. Second, slowing of the heart rate was observed in freely moving mice in the absence of a significant change both in the heart rate variability profile and in the arterial blood pressure, thus arguing against the hypothesis that bradycardia would be caused by dysfunction in the autonomic regulation of the heart rate or could be secondary to a change in the control of the vascular tone. The prolongation of the SAN recovery time also indicates dysfunction in SAN automaticity. Finally, the slowing of pacemaker activity observed in isolated SAN pacemaker cells of Cav3.1−/− mice is consistent with bradycardia being attributable to the lack of I_{Ca,T} in the SAN. The reduction of the mean heart rate measured on freely moving Cav3.1−/− mice is moderate, compared with the slowing of the cycle length in isolated pacemaker cells (10% in freely moving mice and 37% in isolated cells). This difference can be caused by the compensatory adrenergic tone in vivo, which stimulates ionic mechanisms involved in pacemaking, such as Cav1.3-mediated I_{Ca,L}9,10 and I_{f} and RyR-dependent diastolic release of Ca²⁺.11 The observation that the maximal heart rate is comparable in WT and
Ca$_{3.1}^{-/-}$ mice is consistent with this hypothesis. Comparison of mice lacking Ca$_{1.3}^{+}$ and Ca$_{3.1}$ channels (this study) indicates that I$_{Ca,L}$ and I$_{Ca,T}$ play distinct roles in pacemaker activity in vivo. Indeed, knockout of Ca$_{1.3}$ channels reduces the basal and intrinsic heart rate in sedated conditions by approximately 20% and 60%, respectively, compared with the 10% reduction observed in mice lacking Ca$_{3.1}$ channels in both freely moving and block of ANS conditions. These observations suggest that Ca$_{3.1}$ channels contribute to the setting of the basal heart rate but have less impact on the dynamic regulation of pacemaker activity by the ANS. The lack of a significant change in the variability of the RR intervals in Ca$_{3.1}$ mice is consistent with this hypothesis.

We report that the slowing of the AV conduction in Ca$_{3.1}^{-/-}$ mice is caused by dysfunction in the excitability of the AVN. Indeed, intracardiac recordings show a delay in the AH conduction time and AVERP, in the absence of a prolongation of the P wave, which indicates similar conduction velocities in atria from WT and Ca$_{3.1}^{-/-}$ mice. Consistently with ECGs and intracardiac recordings, we found no I$_{Ca,T}$ in atrial myocytes from WT mice. Also, AVN cells display I$_{Ca,T}$ related to CaV3.1 channels because it was no longer detectable in CaV3.1 mice. We did not find significant changes in the HV, QRS, and QT intervals (see Figures 3 and 5 and supplemental Figure III), indicating that the lack of Ca$_{3.1}$ channels does not affect conduction through the His–Purkinje fiber network or the ventricular muscle. This phenotype differs from that of SCN5A$^{-/-}$ mice, which shows prominent alteration of the P wave and the QRS complex duration, stressing the importance of the cardiac

| Telemetric ECG Parameters in Freely Moving WT and Ca$_{3.1}^{-/-}$ Mice |
|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|
|                      | WT                  | Ca$_{3.1}^{-/-}$      |                      |                      |                      |                      |
| Mean HR (bpm)        | 578±14              | 524±11               |                      |                      |                      |                      |
| Daytime Mean HR      | 526±11              | 476±13               |                      |                      |                      |                      |
| Nighttime Mean HR    | 630±18              | 571±12               |                      |                      |                      |                      |
| HR Minimum (40 seconds) | 404±8            | 358±18               |                      |                      |                      |                      |
| HR Maximum (40 seconds) | 775±10           | 742±15               |                      |                      |                      |                      |
| P Wave Duration (ms) | 13±1                | 14±1                 |                      |                      |                      |                      |
| PO Interval (ms)     | 32±1                | 35±0                 |                      |                      |                      |                      |
| QRS Interval (ms)    | 12±1                | 12±0                 |                      |                      |                      |                      |
| QT Interval (ms)     | 56±2                | 52±2                 |                      |                      |                      |                      |
| P                     | 0.012               | 0.015                | 0.022                | 0.039                | NS                    |                      |
| ECG parameters were set at a cycle length of 100 ms. WT, N=6; Ca$_{3.1}^{-/-}$, N=6. HR indicates heart rate.
I\textsubscript{Na} in intraatrial and His–Purkinje conduction. Also in contrast with SCNS5A \textsuperscript{+/−},\textsuperscript{19} Ca\textsubscript{V}3.1\textsuperscript{−/−} mice do not present ventricular tachyarrhythmias. This SCNS5A-mediated I\textsubscript{Na} can possibly compensate for the lack of I\textsubscript{Ca,T} in the His–Purkinje system.

**Pacemaking in Ca\textsubscript{V}3.1\textsuperscript{−/−} Mice**

We describe that inactivation of Ca\textsubscript{V}3.1 channels significantly prolongs the pacemaker cycle length in isolated SAN cells by reducing the slope of the diastolic depolarization, demonstrating the involvement of Ca\textsubscript{V}3.1-mediated I\textsubscript{Ca,T} in the setting of the diastolic depolarization in mouse SAN cells. In an attempt to compare the physiological roles of Ca\textsubscript{V}3.1-mediated I\textsubscript{Ca,T} with that of Ca\textsubscript{V}1.3-mediated I\textsubscript{Ca,L} and I\textsubscript{Na}, we have developed a numerical model of mouse SAN electrophysiology based on previous studies on mouse SAN pacemaking in normal and genetically modified mouse strains (see supplemental Figures IV and V). Our numerical simulations indicate that Ca\textsubscript{V}3.1 channels contribute to pacemaking by activating during the diastolic depolarization for more negative voltages than TTX-sensitive I\textsubscript{Na} and Ca\textsubscript{V}1.3-mediated I\textsubscript{Ca,L} (see supplemental Figure VB and VE). As a consequence, Ca\textsubscript{V}3.1 channels can accelerate the diastolic depolarization rate in a voltage range in which I\textsubscript{Na} is still not activated. This property can explain, at least in part, how Ca\textsubscript{V}3.1 channels can contribute to pacemaking in spite of their relatively low availability at SAN diastolic potentials. An expanded discussion about the development, the features and limitations of our numerical model, and the physiological significance of Ca\textsubscript{V}3.1-mediated I\textsubscript{Ca,T} compared with that of I\textsubscript{Ca,L} and I\textsubscript{Na} is available in the online data supplement.

**Conclusion**

In conclusion, our study demonstrates that Ca\textsubscript{V}3.1 channels contribute to the heartbeat by influencing pacemaking and the AV conduction. To date, the presence of T-type channels in human heart is poorly documented. Molecular analyses, such as dot blots and Northern blots of human heart mRNAs, have identified transcripts for Ca\textsubscript{V}3.1 and Ca\textsubscript{V}3.2\textsuperscript{31,32} T-type channels in cardiac tissues. These channels may play a role in the human SAN and the conduction system. For instance, the causality between congenital heart disease and children showing SAN bradycardia and atrioventricular block induced by maternal autoantibodies against T-type channels in cardiac tissues were recently documented.\textsuperscript{33} There is growing evidence that T-type channels may constitute a promising pharmacological target for the treatment of human diseases, such as epilepsy and chronic pain.\textsuperscript{34} From our observations, T-type channel inhibition would have no deleterious consequences in cardiac physiology. It is, therefore, tempting to speculate that selective blockers of Ca\textsubscript{V}3.1 channels may hold promise for the therapeutic management of the cardiac diseases that require moderate heart rate reduction, such as cardiac ischemia and coronary heart disease.

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Expanded Material and Methods

Mice handling was done in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH publication no. 85-23, revised 1996), and European directives (86/609/CEE) and every effort was made to minimize animal suffering and the number of animals used.

Results are presented as the mean ± the standard error of the mean (SEM); n indicates the number of observations. Statistical significance was assessed by the unpaired Student’s t test. A value of $P<0.05$ was considered as statistically significant and n.s. stands for not significant. N indicates the number of mice used in indicated experiments.

Electrophysiology on isolated SAN, AVN and RA and cells

SAN tissue samples and pacemaker cells were isolated from age- and sex-matched 129 sv WT or CaV3.1−/− adult mice as previously described. Myocytes from the right atrium were isolated using a similar procedure as described for SAN cells. The AVN was identified by using landmarks as in 2. Individual AVN cells were isolated by applying the same digestion/dissociation procedure as for SAN cells. Ca²⁺ currents were recorded at 26°C with the conventional whole-cell patch-clamp technique using extracellular 2 mM Ca²⁺ and analyzed as described previously. The cell access resistance in these recording conditions was around 10 MΩ, series resistance was evaluated at the peak of $I_{Ca}$ and could be corrected up to 75%. Capacitative transients were not compensated electronically. Ca²⁺ currents were recorded using an extracellular solution containing (in mmol/L): TEA-Cl, 130; CaCl₂, 2; MgCl₂, 1; 4-aminopyridine, 10; HEPES, 25; (adjusted to pH 7.4 with TEAOH). Whole-cell pipettes were filled with a solution containing (mM) CsCl, 130; EGTA, 10; HEPES, 25; Mg-ATP 3 and Na-GTP 0.4 (pH at 7.2 with CsOH). Pacemaker activity of SAN cells was
recorded at 35°C under perforated-patch conditions using β-escin \(^4\) into the pipette solution at a final concentration of 30 µM. The pipette solution was (in mmol/L): KCl, 130; NaCl, 10; ATP-Na\(^+\) salt, 2; creatine phosphate, 6.6; GTP-Mg\(^{2+}\), 0.1; CaCl\(_2\), 0.04 (pCa 5.7); HEPES-KOH, 10; (adjusted to pH 7.2 with KOH). The extracellular solution contained (in mmol/L): NaCl, 140; KCl, 5.4; CaCl\(_2\), 1.8; MgCl\(_2\), 1; Hepes-NaOH, 5; and D-glucose, 5.5; (adjusted to pH 7.4 with NaOH). Myocytes from the RA were isolated using a similar procedure as described above and according to a previous study \(^5\). All voltage values were corrected for the appropriate liquid junction potential. All electrophysiological data were recorded and analyzed using the pCLAMP suite ver 9.0 (Axon Instruments).

**Electrocardiograms (ECGs) on anesthetized mice**

Adult mice were anesthetized with etomidate (30 mg/kg i.p.). Body temperature was maintained at 36°C-37°C with a warming pad controlled by a rectal temperature probe. Surface ECGs were recorded with 25-gauge subcutaneous electrodes on a computer through an analog-digital converter (IOX 1.585, EMKA Technologies) for monitoring and later analysis (ECG Auto 1.5.7, EMKA Technologies). ECG channels were filtered between 0.5 and 250 Hz. Reported measurements were averaged from 3 consecutive PQRST complexes in lead I. Criteria used for interval measurements have been described elsewhere \(^6\). The QT interval was corrected for heart rate using the formula, QTc=QT/(RR/100)^{1/2} established for mice with QT and RR expressed in ms \(^7\). ECGs were first recorded under baseline conditions and then 10 min after atropine sulfate (0.5 mg/kg i.p.) and propranolol (1 mg/kg i.p.) injection to block the autonomic nervous system (ANS).
Intracardiac recording and pacing.

**Mouse preparation.** Animals were anesthetized with an intra-peritoneal injection of etomidate (8 mg/kg) and pentobarbital (30 mg/kg). Body temperature was maintained at 36°C-37°C with a warming pad controlled by a rectal temperature probe. Under sterile conditions, an octapolar 2F catheter with an electrode spacing of 0.5 mm (Cordis Webster®) was introduced into the right atrium and ventricle through the right internal jugular vein. Using this catheter, simultaneous atrial and ventricular pacing and recording were performed. His bundle activity was also recorded when detected. Intracardiac electrograms were filtered between 30 and 500 Hz. Surface ECG (lead I) and intracardiac electrograms were recorded to a computer through an analog to digital converter (IOX 1.585, EMKA Technologies) for monitoring and off-line analysis and measurement. Intracardiac pacing was performed with a Biotronik® UHS20 stimulator, modified by the manufacturer to pace at short coupling intervals.

**Electrophysiological study protocols.** Standard pacing protocols were used to determine the electrophysiologic parameters. Sinus node function was evaluated by measuring the rest sinus cycle length and the sinus node recovery time. After a period of normal sinus rhythm (RR interval values of 109± 5 ms for WT and 120 ± 9 ms for Ca₃.1Δ⁻/⁻ mice; NS; n=9 and 7 respectively), atrial pacing was applied for a period of 30 s at cycle lengths of about 80% of the intrinsic sinus cycle length (78% ± 2% in WT mice, n = 9; 77% ± 1% in Ca₃.1Δ⁻/⁻ mice, n = 7; NS). For each pacing cycle length, sinus node recovery time (SNRT) was determined as the longest pause from the last paced atrial depolarization to the first sinus return cycle. Here we report cSNRT corrected by subtracting the intrinsic sinus cycle length from the recovery interval. Anterograde AV nodal conduction properties were assessed by pacing the atrium at rapid rates and determining the maximum cycle length resulting in Wenckebach phenomenon.
Refractory periods were determined by delivering an 8-stimulus drive train (S1) at a cycle length of 100 ms followed by a premature stimulus (S2) progressively decremented in 2 ms intervals. Atrial, atrioventricular nodal and ventricular effective refractory periods (AERP, AVERP and VERP, respectively) were defined as the longest S1-S2 coupling interval for each region that failed to generate a propagated beat with S2.

**ECG recording in conscious mice**

For long-term ECG recording, adult male mice were anesthetized with etomidate (35 mg/kg i.p.). A midline incision was made on the back along the spine to insert a telemetric transmitter (TA10EA-F20, Data Sciences International) into a subcutaneous pocket with paired wire electrodes placed over the thorax (chest bipolar ECG lead). Local anesthesia was obtained with lidocaine (1%) injected subcutaneously at the sites of electrodes and transmitter implantation. Experiments were initiated at least 8 days after recovery from surgical implantation. Mice were housed in individual cages with free access to food and water and were exposed to 12-hour light/dark cycles (light, 8:30 AM to 8:30 PM) in a thermostatically controlled room. ECG signals were computer-recorded with the use of a telemetry receiver and an analog-to-digital conversion data acquisition system for display and analysis by Dataquest™ A.R.T.™ software (Data Sciences International). Heart rate values were determined from RR intervals averaged every 10 seconds. Mean heart rate values were obtained in each mouse for an overall 24-hour period from 8:30 AM to 8:30 PM and for the corresponding 12-hour light and dark periods. Maximal and minimal heart rate values were determined on 40-second periods during dark and light periods respectively. ECG parameters were measured at a fixed RR interval of 100 ms with ECG Auto 1.5.7 software. Spectral analysis using a fast Fourier transformation algorithm on sequences of 512 points was performed using the ECG Auto 1.5.12.10 software (EMKA Technologies) on RR data series.
recorded between 11 AM and 2 PM. The area under the curve was calculated for the ultra-
low-frequency (ULF: 0 to 0.026 Hz), very-low-frequency (VLF: 0.026 to 0.32 Hz), low-
frequency (LF: 0.32 to 1.2 Hz), and high-frequency (HF: 1.2 to 3.2 Hz) bands, as previously
defined in the mouse species\textsuperscript{8}.

**Measurements of the blood pressure**

Systolic and diastolic blood pressures were measured on conscious mice using a computerized
tail-cuff method (BP-2000, Visitech Systems, Apex, NC). Following a 7-day acclimation
period, mice were trained for 6 days on the equipment prior performing measurements.
Animals were maintained restrained at 37°C in the dark and tail cuffs were placed on the tail
of each of the mice. In order to acclimate the mice, 10 preliminary measurements were
performed. Blood pressure was then determined and recorded. Mice were tested over 3 days
and 10 measurements per day were collected. The final systolic blood pressure value was
calculated as the average of 30 measurements.

**RT-PCR Analysis**

For RT-PCR analysis, total RNAs from sino-atrial node (SAN), the right atrium (RA), the left
ventricle (LV) and the atrio-ventricular node (AVN) adult mice were extracted using the
SNAP\textsuperscript{TM} Kit (Invitrogen). Reverse transcription (RT) was performed with total RNA using
random primers and Superscript II RNase H reverse transcriptase (Invitrogen). Following RT,
the cDNAs for \( \text{Ca}_v3.1, \text{Ca}_v3.2 \) and \( \text{Ca}_v3.3 \) were amplified using the following set of primers :
\( \text{Ca}_v3.1-\text{F} \ 5'\text{-GTCTCCGCACGGTCTGTAAC-3}' \ (\text{NM}_009783, \text{nucleotide (nt) 329}), \text{Ca}_v3.1-
\text{R}, \ 5'\text{-CCACAGCAAAGAAGGCAAG-3}' \ (\text{nt 499, expected size 170bp}), \text{Ca}_v3.2-\text{F} \ 5'-
\text{TGGGAACGTGCTTCTTCTCT-3}' \ (\text{NM}_021415, \text{nt 690}), \text{Ca}_v3.2\text{R}, \)
GGGGATGTGTGAGCATTTCT (nt 918, expected size 228bp), CaV3.3-F 5’-GACACCACTCCCCACACACT-3’(XM_139476, nt 3250), CaV3.3-R, CCTCCTGTTCCTCCTCCTTC-3’ (nt 3487, expected size 237 bp). PCR was performed in a final volume of 25 µl containing 2 µl of reverse transcription reaction, 10 pmol of each primer, 2.5 mM each dNTP (Pharmacia), 1.5 mM MgCl₂ and 1 unit of Taq polymerase (Sigma) in a MJ Research, Inc Thermal Cycler with the following cycling protocol: after 3 min at 94°C, 35 cycles (94°C, 45s; annealing temperature, 45s; 72°C, 35s) of PCR were performed followed by a final elongation period of 10 min at 72 °C. Annealing temperatures were as follows: 57°C for CaV3.1, 55°C for CaV3.2 and 59°C for CaV3.3. To visualize the presence and size of the amplified fragments, 10 µl of the PCR products were electrophoretically separated on an ethidium bromide-stained 2% agarose gel.

**Quantitative RT–PCR**

CaV3 mRNA expression was determined by quantitative RT-PCR using a real time TaqMan PCR technology (ABI Prism 7000, Applied Biosystems) using the following sets of specific primers for CaV3.1, CaV3.2 and CaV3.3 and hypoxanthine guanine phosphoribosyl transferase (HPRT) : CaV3.1 : Q-CaV3.1-F 5’-CCAAGCAACGGGAGAGTCA-3’ (NM_009783, nt 1310) and Q-CaV3.1-R 5’-GCTAGCATTGGACAGGAATCG-3’ (nt 1371, expected size 61bp) ; CaV3.2 : Q-CaV3.2-F 5’-CAGCGGCTACGCCTATGC-3’(NM_021415, nt 3230) and Q-CaV3.2-R 5’-CAAAAGAGTGTGGGCCATGTC-3’(nt 3294, expected size 65bp) ; CaV3.3 : CaV3.3-F 5’-CCTTTTTGCTCTTGTGTTTTCTG-3’ (XM_139476 nt 1106), CaV3.3-R 3’-ACTTTAGGAGGCATCATTATATTCC-3’ (nt 1219, expected size 113bp) and HPRT : Q-HPRT-F 5’-GCAGTACAGCCCCAAATGG-3’ (NM_013556, nt 540), and Q-HPRT-R 5’-GGTCCCTTTTCACCAGCAAGCT-3’ (nt 591, expected size 51bp). Experiments were performed using 1X SYBR Green PCR Master Mix TaqMan PCR buffer from Applied
Biosystems and 300 nM of each primer. The cycling conditions included a hot start for 10 min at 95°C, followed by 40 cycles at 95°C for 15s, and 60°C for 1 min. All primers sets were tested under the Taqman PCR conditions using mouse genomic DNA as template. In all cases, the presence of a single product of the appropriate size was controlled by agarose gel electrophoresis (Figure 2B). Each amplification was normalized to HPRT, the reference mRNA. mRNA samples that were incubated without reverse transcriptase during cDNA synthesis showed no amplification (negative controls).

**Numerical modelling of mouse SAN cell pacemaker activity**

Numerical modelling of pacemaker activity was performed according to the central SAN model by Zhang et al. 9. We have chosen this model since it fairly reproduces basal pacemaking in spite of the absence of experimental data on intracellular ionic concentration and homeostasis, which is in fact the case for mouse SAN pacemaker activity. Also, this model has been developed to account for pacemaking in small cells likely from the center of the node and having similar capacitance than that used in our study (21 pF). The original SAN model has been appropriately modified in accordance to experimental data obtained by different groups in mouse pacemaker cells. To this aim, we have added equations to account for the presence of Ca\(_{1.3}\)- and Ca\(_{1.2}\)- mediated \(I_{Ca,L}\), according to previous studies on Ca\(_{1.3}^{+/−}\) mice 3,10. These current components have been separated with respect to their differential steady state parameters for activation and inactivation. Similarly, TTX- sensitive and TTX- insensitive \(I_{Na}\) currents have been included in the model according to experimental results on isolated mouse SAN cells by Lei et al. 11. Ca\(_{3.1}\)- mediated \(I_{Ca,T}\) was modelled to match voltage clamp results obtained in Fig. 1 and in ref. 3. Also, equations for the \(I_{K1}\) and \(I_{st}\) were added according to a previous report on mouse SAN cells 12.

Numerical simulations were performed by employing the XPPAUT software, freely available at [http://www.pitt.edu/~phase/](http://www.pitt.edu/~phase/). Due to the absence of experimental data from mouse SAN
cells, all equations and parameter values for background voltage-independent currents, ionic pumps and exchangers were kept as in the original model of rabbit central SAN cells \(^9\) (see also the online supplementary table 1). The complete list of parameter values and experimental current densities used for simulations are shown in the online supplementary table 1 and 2, respectively. The list of initial values is shown in the online supplementary table 3.

**Modelling of \(I_{Ca,T}\)**

A Hodgkin-Huxley (HH) model of \(Ca_{v3.1}\)-mediated \(I_{Ca,T}\) was constructed according to the equations described in Destexhe & Huguenard \(^13\). Steady state parameters for activation were taken from our \(I_{Ca,T}\) records in SAN cells from WT mice. Steady-state inactivation parameters of \(I_{Ca,T}\) were from our previous study on SAN cells from \(Ca_{v1.3^{-/-}}\) mice \(^3\) which allow precise measurement of \(I_{Ca,T}\) at negative test potentials (see also ref. \(^14\)). Activation and inactivation time constants were measured in sample records from WT mice at 36 °C (data not shown) and compared with records of \(I_{Ca,T}\) from \(Ca_{v1.3^{-/-}}\) mice \(^3\). For \(I_{Ca,T}\) reactivation kinetics, we used experimental records obtained from recombinant \(Ca_{v3.1b}\) channels \(^15\) which are expressed in the heart \(^16,17\) and show the greatest similarity in steady state properties with the native mouse SAN \(I_{Ca,T}\).

**Modelling of \(I_{Ca,L}, I_{Na}\) and \(I_{st}\)**

For modelling of \(I_{Ca,L}\), we have used the same equations as in Zhang et al \(^9\). According to experimental evidence from SAN cells of \(Ca_{v1.3^{-/-}}\) mice, we have assumed that the total whole-cell \(I_{Ca,L}\) is composed by both \(Ca_{v1.3}\) and \(Ca_{v1.2}\) channels. Two independent groups of equations were used for \(Ca_{v1.3}\)- and \(Ca_{v1.2}\)-mediated \(I_{Ca,L}\). Activation and inactivation steady-state parameters used in the model, as well as current densities of \(Ca_{v1.3}\)- and \(Ca_{v1.2}\)-
mediated $I_{Ca,L}$ are from our previous study on Ca v1.3$^{-/-}$ mice. For modelling $I_{Na}$, we have used equations as in the atrial cell model by Nygren and co-workers. We have assumed that the total whole-cell $I_{Na}$ is composed by TTX-sensitive ($I_{Na}$) and TTX-resistant ($I_{Nar}$) $I_{Na}$ according to Lei et al. Steady-state activation/inactivation parameters as well as the relative current densities of $I_{Na}$ and $I_{Nar}$ for a SAN cell of 21 pF are from Fig. 3F and 2K in Lei et al, respectively. The $I_{st}$ current was also added in our model. To this aim we have employed the HH empirical model of $I_{st}$ from Shinagawa et al. $I_{st}$ densities and activation are from mouse SAN cells according to ref. 12.

**Modelling of K$^+$ currents and $I_f$**

All voltage-dependent K$^+$ currents included in the original model by Zhang et al. have been included in our calculations, except for $I_{K1}$, that we have added to account for the observed $I_{K1}$ in mouse SAN cells. $I_{K1}$ was calculated according to equations in Nygren et al. $I_{K1}$ density was taken from Cho et al. For modelling of $I_{Kr}$, we used the equations from Kurata et al. These equations, fairly reproduced experimental results on mouse SAN pacemaker cells published in Clark et al. The conductance of all other voltage-dependent K$^+$ currents included by Zhang et al., have been kept as in the original model of rabbit central SAN cells (see online supplementary table 1). For $I_f$, we used the same equations as in Zhang et al. To account for mouse SAN cells, $I_f$ density and activation were adjusted according to experimental results by Mangoni & Nargeot.

**Simulation of mouse pacemaker activity**

We have developed a model of mouse SAN electrophysiology to gain insights into the behaviour of $I_{Ca,T}$ during pacemaking (supplementary Figure 4, 5). To this aim, we have adapted the model for central SAN cells by Zhang and co-workers to account for published
data on isolated mouse SAN cells ionic conductances (see the Methods section). The model
takes into account the expression of Ca\textsubscript{v}1.3- and Ca\textsubscript{v}1.2-mediated $I_{Ca,L}$ \cite{3,10} as well as TTX-
sensitive and TTX-resistant $I_{Na}$ \cite{11}. The model also includes $I_{st}$ current according to a recent
study on mouse SAN cells by Cho and coworkers \cite{12}. $I_{Kr}$ was also modelled to account for
experimental data in Clark et al \cite{21}. $I_{K1}$ was also included in the model, since this current has
been consistently found in mouse SAN cells \cite{12} (M. Mangoni, unpublished observations. As
shown in supplementary Figure 4A, our model fairly reproduces current densities and
waveforms experimentally observed in voltage-clamp conditions. $I_{Ca,T}$ density and voltage
dependence was set according to experimental parameters (Figure 1B). The density of Ca\textsubscript{v}1.3-
mediated $I_{Ca,L}$ was set to 10 pA/pF (see supplementary table 2). This value is higher than that
found experimentally in this study (5-6 pA/pF, see Figure 1). We have preferred to assume
similar densities for Ca\textsubscript{v}3.1 and Ca\textsubscript{v}1.3-mediated currents since Ca\textsubscript{v}1.3 channels are already
partially inactivated at a Hp of -55 mV \cite{3} (see supplementary Figure 4B), leading to a possible
underestimation of the absolute $I_{Ca,L}$ density compared to $I_{Ca,T}$ in voltage-clamp conditions.
Furthermore, $I_{Ca,L}$ densities higher than 6 pA/pF can be found on isolated SAN cells and
activation of the $\beta$-adrenergic receptor strongly stimulates Ca\textsubscript{v}1.3- and Ca\textsubscript{v}1.2-mediated $I_{Ca,L}$
\cite{3}. As to TTX-sensitive and TTX-resistant $I_{Na}$, our modelling work reproduced the data by Lei
and co-workers \cite{11}. Steady-state voltage dependencies for activation and inactivation of both
$I_{Na}$ current components are shown in the supplementary Figure 4C. The density of TTX-
sensitive $I_{Na}$ was chosen to match that in reported action potential clamp experiments, thereby
yielding a current amplitude between 10 and 20 pA (20 pA in our simulations) during the
diastolic depolarisation (see Figure 3E of ref. \cite{11}). The density of TTX-resistant $I_{Na}$ was set as
for cells having capacitance of 30 pF in Lei et al. \cite{11}. This density has been measured in low-
Na\textsuperscript{+} conditions. This can lead to some underestimation of TTX-resistant $I_{Na}$ in our
calculations even if the presence of this current in SAN cells of less than 30 pF has not been
directly demonstrated. However, it was necessary to include TTX-resistant $I_{Na}$, since it has been demonstrated that $SCN5A^{+/}$ mice show moderate bradycardia and that TTX-resistant $I_{Na}$ participate to the setting of the normal SAN function.

When parameters in supplementary table 1 are applied, the model generates a basal rate of 278 bpm (cycle length is 216 ms, Supplementary Figure 5A), with a MDP of -65 mV and a takeoff potential of about -45 mV. Abolition of Ca$_{v}$3.1-mediated $I_{Ca,T}$ from the model induced 8% rate reduction, by slowing the diastolic depolarisation. Upon deactivation of large $I_{Kr}$, $I_{f}$ is the first voltage-dependent inward current to activate in the diastolic depolarisation (supplementary Figure 5C), $I_{nf}$ is also present in the same range of voltages and drives a residual fraction of current which has not completely deactivated after repolarization (supplementary Figure 5D). Ca$_{v}$3.1-mediated $I_{Ca,T}$ starts to activate at about -60 mV and peaks at -40 mV (supplementary Figure 5D, E). As a comparison, TTX-sensitive $I_{Na}$ activates during the late phase of the diastolic depolarisation at about -50 mV and peaks around -30 mV (supplementary Figure 5E). This observation is consistent with the behaviour of TTX-sensitive $I_{Na}$ in action potential clamp experiments (see Figure 3 E, F in Lei et al. and the related discussion). Our simulations predict that Ca$_{v}$3.1-mediated $I_{Ca,T}$ can contribute to the diastolic depolarisation by activating earlier than the fast TTX-sensitive and TTX-resistant $I_{Na}$. (supplementary Figure 5B). The negative activation range of Ca$_{v}$3.1-related $I_{Ca,T}$ indicate that TTX-sensitive $I_{Na}$ cannot compensate for the lack of $I_{Ca,T}$ in Ca$_{v}$3.1$^{-/-}$ mice, at least under basal conditions. Ca$_{v}$1.3-mediated $I_{Ca,L}$ is predicted to be present throughout the diastolic depolarisation phase (supplementary Figure 5B, D). This persistent component is likely to be due to both slow inactivation kinetics of Ca$_{v}$1.3 channels and/or to the predicted window Ca$_{v}$1.3-mediated $I_{Ca,L}$ (supplementary Figure 4B). Incomplete inactivation of Ca$_{v}$1.3-mediated $I_{Ca,L}$ has also been observed upon application of pacemaker action potential waveforms to HEK cells expressing recombinant human Ca$_{v}$1.3 channels. This persistent $I_{Ca,L}$ component
contribute to the diastolic depolarisation by summing to Ca_v3.1-mediated \( I_{Ca,T} \), \( I_{st} \) as well as to both TTX-sensitive and –resistant components of \( I_{Na} \) close to the cell takeoff potential. For this reason, Ca_v1.3-related \( I_{Ca,L} \) is the major voltage-dependent Ca^2+ current contributing to mouse SAN pacemaking. In our model, \( I_{st} \) is activated in the same voltage range as \( I_{Ca,T} \). Together with Ca_v1.3-related \( I_{Ca,L} \), \( I_{st} \) is strongly sensitive to β-adrenergic agonists^{12}. These currents can thus constitute two major mechanisms of compensation of the lack of Ca_v3.1 channels at maximal heart rates which is presumably driven by strong β-adrenergic input on the SAN (see table1). Compared to Ca_v1.3 and TTX-sensitive channels, Ca_v3.1 channels are strongly inactivated at positive MDPs. We can expect that the relative contribution of Ca_v3.1-mediated \( I_{Ca,T} \) to pacemaking will depend upon the cell MDP as well as the degree of stimulation of currents by the β-adrenergic/cAMP-dependent signalling pathway. Accordingly, switching the model MDP to more positive voltages (supplementary Figure 5F), reduces the effect of \( I_{Ca,T} \) abolition on the calculated pacing rate.

We have also directly measured in SAN cells the level of Ca_v3.1-mediated \( I_{Ca,T} \) at a test potential of -50 mV from different HP ranging from -70 to -55 mV. To this aim, we have employed WT SAN cells in which \( I_{Ca,L} \) did not show detectable activation at -50 mV. In these conditions, averaged Ca_v3.1-mediated \( I_{Ca,T} \) peak (isochronal) densities were 0.66±0.3 pA/pF from a HP of -65 mV, 0.18±0.065 pA/pF from a HP of -60 mV and 0.044±0.026 from a HP of -55 mV (N=2, n=5, peak \( I_{Ca,T} \) =7.5±2 pA/pF at -30 mV, Hp=-90 mV). As a comparison, our model predicts in voltage-clamp conditions 1.9 pF peak \( I_{Ca,T} \) from a HP of -65 mV, 0.47 pA/pF from a HP=-60 mV and 0.11 pA/pF from HP=-55 mV (\( I_{Ca,T} \) peak density 10 pA/pF).

**Interest of the model study**

The development of a numerical model of mouse SAN electrophysiology based on experimental data coming from normal and genetically-modified mouse strains constitute an
important step toward understanding the physiological role and significance of Ca\textsubscript{v3.1}-mediated $I_{\text{Ca,T}}$ compared to other ionic channels. The goal of our numerical simulations is to define the voltage range of activation as well as the relative level of $I_{\text{Ca,T}}$ during pacemaker activity with respect to other ionic currents involved in the genesis and regulation of automaticity. Calculations indicate that Ca\textsubscript{v3.1}-mediated $I_{\text{Ca,T}}$ can contribute to the diastolic depolarisation by activating early in the diastolic depolarisation phase together with $I_{\text{st}}$. The contribution of Ca\textsubscript{v3.1}-mediated $I_{\text{Ca,T}}$ to overall pacemaking is predicted to be moderate, an observation which is experimentally verified. Our calculations are thus also important for indicating how Ca\textsubscript{v3.1} channels can contribute to the diastolic depolarisation in spite being partly inactivated at positive MDPs. The physiological significance of Ca\textsubscript{v3.1}-related $I_{\text{Ca,T}}$ can also be linked to the presence of large deactivating $I_{K_r}$ at the end of repolarization. Indeed, the presence of $I_{\text{Ca,T}}$ can favour the development of the diastolic depolarisation in a voltage range in which large outward $K^+$ currents are still present. In our numerical model, we did not observe a change in the upstroke phase upon abolition of Ca\textsubscript{v3.1}-related $I_{\text{Ca,T}}$ (supplementary Figure 5A). Consistently with the work of Lei et al.\textsuperscript{11}, abolition of TTX-sensitive and resistant $I_{\text{Na}}$ affected the SAN rate, as well as the takeoff potential and the upstroke phase in our simulations (not shown). These observation indicate that Ca\textsubscript{v3.1}-related $I_{\text{Ca,T}}$ and $I_{\text{Na}}$ play complementary and distinct roles in mouse SAN pacemaking.

Each numerical model of pacemaking has its internal limitations. In our model, limitations are linked to the assumption that internal Ca\textsuperscript{2+} concentration remains constant and to the available data on mouse SAN automaticity. Indeed, even if detailed modelling of diastolic release of Ca\textsuperscript{2+} have been developed for rabbit SAN cells\textsuperscript{23}, no quantitative experimental data are available on this newly described pacemaker mechanism in mouse SAN cells. Consequently, inclusion of RyR-dependent Ca\textsuperscript{2+} release in our model would be based only on assumptions about parameters scaling. To this respect, the basal calculated rate is about half than that
observed in freely-moving mice (table1). This discrepancy can be in part ascribed to $I_{Kr}$
density that has been set to account for data coming from larger cells and to the inclusion of
$I_{Ks}$ and $I_{sus}$ in the model. Furthermore, our model does not include diastolic release of Ca$^{2+}$
which has been shown to strongly accelerate the diastolic depolarisation rate in rabbit SAN
cells$^{24,25}$ and in numerical models of pacemaking$^{23,26}$. Finally, the basal adrenergic tone in
vivo which stimulates $I_{Ca,L}$, $I_f$, $I_{st}$ and diastolic Ca$^{2+}$ release beyond their basal levels can also
explain the difference between the basal model rate and that observed in freely-moving mice.
The lack of experimental data on diastolic Ca$^{2+}$ release in mouse SAN cells also prevented us
to get insights into pacemaking during activation of the $\beta$-adrenergic receptor. Nevertheless,
our model can be updated as new experimental data on mouse SAN will be available.

The observation that the reduction of pacing rate is higher in SAN cells from Ca$_{v}3.1^{-/-}$ mice
(see Figure 4 A, B) than that predicted in our simulations suggests that the contribution of
Ca$_{v}3.1$-mediated $I_{Ca,T}$ to the diastolic depolarisation may also be linked to intracellular Ca$^{2+}$
signalling. Indeed, Huser et al.$^{27}$ have reported that in latent pacemaker cells, T-type channels
could trigger Ca$^{2+}$ release, since diastolic Ca$^{2+}$ sparks were generated in a voltage range
compatible with T-type channel activation and were blocked by 50 µM Ni$^{2+}$. This view has
been challenged in a recent work on diastolic Ca$^{2+}$ release in primary rabbit SAN pacemaker
cells$^{25}$ in which no change in Ca$^{2+}$ sparks were reported in the presence of 30 µM Ni$^{2+}$. The
fact that Ca$_{v}3.1$ channels are only partly inhibited at 50 µM Ni$^{2+}$ indicate the necessity to
investigate if SAN Ca$_{v}3.1$ channels are coupled to Ca$^{2+}$ release during diastolic depolarization
in the mouse SAN. On the other hand, a species-dependent influence on the functional
expression of Ca$_{v}3.2$ in the adult heart cannot be excluded. For instance it has been reported
that $I_{Ca,T}$ was strongly inhibited by 50 µM Ni$^{2+}$ in cat latent pacemaker cells$^{27}$, thus rising the
possibility of functional Ca$_{v}3.2$ channels in these cells.
References cited in the supplementary data:


Online Supplementary Figure Legends

Online supplementary Figure 1:

No difference in capacitance and cell morphology between WT and Ca₃.1⁻/⁻ cardiac cells. Electrophysiological recordings of SAN cells from WT and Ca₃.1⁻/⁻ mice animals showed no change in the cell capacitance (19±2 pF N=13, n=17 and 20±1 pF N=12, n=18, respectively). Consistently, the averaged upstroke velocities in action potentials recorded (see Fig. 4 in the main text) were 20±5 V/S (N=5, n=14) and 21±5 V/S, (N=4, n=15) in SAN cells from WT and Ca₃.1⁻/⁻ mice. These values are consistent with the leading mouse SAN pacemaker cells presumably from the center of the SAN. In addition, no difference in their morphology could be identified (supplementary Figure 1A). Similarly, inactivation of Ca₃.1 induce no change in capacitance for AVN cells (17±1 pF N=5, n=7 and 18±4 pF, N=4, n=7; supplementary Figure 1B) and for RA cells (33.3±1.4 pF and 32.7±2.3 pF N=3, n=8; supplementary Figure 1C).

Online supplementary Figure 2.

Expression of T-type Ca²⁺ channel isotype mRNAs in wild-type (+/+ ) and Ca₃.1⁻/⁻ (-/-) mouse heart. Transcripts for the Ca₃.1 and Ca₃.2 subunits, but not for the Ca₃.3 subunit, were detected in the SAN, as compared to that obtained with hippocampus mRNA used as a positive control (Supplementary Figure 2A). The disruption of the Ca₃.1 gene was assessed by using a set of primers overlapping from the first to the third exon. Expectedly, no Ca₃.1 transcripts were detectable in Ca₃.1⁻/⁻ mice since the forward primer used to identify Ca₃.1 transcripts was comprised within exon 1 that was deleted to generate a null allele for cacna1g. Because RT-PCR experiments indicated that Ca₃.1 transcripts were present in
various cardiac tissues, real-time TaqMan PCR was developed to quantitatively analyze T-type channel mRNA expression levels in various cardiac areas: SAN, sino-atrial node; AVN, atrioventricular node; RA, right atrium; LV, left ventricle (Supplementary Figure 2B). mRNA contents were normalized according to HPRT mRNA contents and the results are expressed as $2^{-\Delta Ct} \times 100$. Each value represents the mean of at least 6 experiments in triplicate out of 3 independent RT samples. The upper panel shows the corresponding gels (Mw: molecular weight). The highest amounts of Ca$_{v}3.1$ mRNA were detected both in the SAN and in the AVN, while the amount of Ca$_{v}3.1$ mRNA in the RA and in the LV was significantly lower. Real-time PCR also confirmed the absence of Ca$_{v}3.3$ mRNA in all cardiac tissues while Ca$_{v}3.2$ mRNA was detected in mouse cardiac tissues, especially in the RA. We found mRNAs for both Ca$_{v}3.1$ and Ca$_{v}3.2$ channels in the adult mouse SAN, AVN and the RA (see supplementary Figure 2). Our RT-PCR results are consistent with in situ hybridisation data describing lower Ca$_{v}3.2$ mRNA levels as compared to Ca$_{v}3.1$ 29. Interestingly, several recent studies have revealed that after birth only $I_{Ca,T}$ related to Ca$_{v}3.1$ channels is detectable, while both Ca$_{v}3.1$ and Ca$_{v}3.2$ channels are expressed in the foetal heart 17,30-32. These data suggest a developmental isotype switch in the mouse heart with embryonic $I_{Ca,T}$ corresponding to a mix of Ca$_{v}3.2$ and Ca$_{v}3.1$ channels, while after birth only Ca$_{v}3.1$ channels would be functionally expressed.

**Online supplementary Figure 3.**

Relation between the QT and RR ECG intervals recorded in sedated WT (open circles) and Ca$_{v}3.1^{/-}$ mice (filled circles). Each data point represents one mouse.
Online supplementary Figure 4.

Voltage-dependent properties of ionic currents calculated using the mouse SAN electrophysiology model. (A). Voltage clamp traces of voltage-dependent ionic currents (B). Activation and inactivation curves for Cav3.1 ($d_T, f_T$), Cav1.3 ($d_{LD}, f_{LD}$) and Cav1.2 ($d_L, f_L$) channels used in the model, as indicated in the panel legend. (C). Activation and inactivation curves for TTX-sensitive and TTX-resistant $I_{Na}$, as indicated in panel legend. Abbreviations used in panels B, C for gating variables are referred in supplementary table 3.

Online supplementary Figure 5.

Simulated mouse SAN action potentials and underlying ionic currents. (A). Effect of abolishing Cav3.1-mediated $I_{Ca,T}$ on a simulated SAN action potential obtained with parameters indicated in supplementary tables 1-3. (B). Activation of Cav3.1-mediated $I_{Ca,T}$ compared with Cav1.3, Cav1.2 and TTX-sensitive and resistant $I_{Na}$ as indicated in the panel legend. (C). Activation during the pacemaker cycle of voltage-dependent $K^+$ currents, together with $I_{K1}$ and $I_f$. (D). Current-to-voltage relationship of Cav3.1, Cav1.3 and Cav1.2 channels during the pacemaker cycle shown in (A). The predicted $I_s$ current is also shown here. (E). Current to voltage relationships comparing Cav3.1 channels to TTX-sensitive and TTX-resistant $I_{Na}$ as indicated in the panel legend, for the pacemaker cycle in (A). (F). Effects of Cav3.1 abolition in a simulated SAN action potential obtained by setting $I_{K1}$ density to zero and by lowering the conductance of $I_{Kr}$ to 0.0018 $\mu$S.
**Online Supplementary table 1.**

*Parameter values of the mouse SAN cell model.* Generally, abbreviations are the same as in the original model by Zhang et al. Other abbreviations define the conductance of $I_{n}(g_{n})$ and that of $I_{K1}(g_{K1})$.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Absolute value</th>
<th>Abbreviation</th>
<th>Absolute value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{m}$</td>
<td>21 pF</td>
<td>$Pinac$</td>
<td>-15</td>
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<tr>
<td>$d_{NaCa}$</td>
<td>0.0001</td>
<td>$F_{K,r}$</td>
<td>0.4</td>
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<tr>
<td>$E_{Ca,L}$</td>
<td>46.4 mV</td>
<td>$g_{to}$</td>
<td>0.00049 µS</td>
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<tr>
<td>$E_{Ca,T}$</td>
<td>45 mV</td>
<td>$g_{sus}$</td>
<td>0.000065 µS</td>
</tr>
<tr>
<td>$G_{Na}$ (TTX resistant)</td>
<td>7.5x10^{-7} µS</td>
<td>$g_{K,s}$</td>
<td>0.000518 µS</td>
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<tr>
<td>$I_{Na}V_{0.5act}$</td>
<td>-44 mV</td>
<td>$g_{i,Na}$</td>
<td>0.002 µS</td>
</tr>
<tr>
<td>$I_{Na}V_{0.5inact}$</td>
<td>-65 mV</td>
<td>$g_{i,K}$</td>
<td>0.002 µS</td>
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<tr>
<td>$G_{Na}$ (TTX sensitive)</td>
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<td>$g_{b,Na}$</td>
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<tr>
<td>$g_{Ca,L}$</td>
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<td>$[Na^{+}]_o$</td>
<td>140 mM</td>
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<tr>
<td>$g_{Ca,T}$</td>
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<td>$[Na^{+}]_i$</td>
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<tr>
<td>$I_{Ca,T}V_{0.5act}$ (Ca,3.1)</td>
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<tr>
<td>$g_{st}$</td>
<td>0.017 µS</td>
<td>$[K^{+}]_o$</td>
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<td>$[K^{+}]_i$</td>
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<tr>
<td>$g_{K,r}$</td>
<td>0.0022 µS</td>
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Online supplementary table 2.

Current densities. The table shows the experimental ionic current densities used in modelling mouse SAN cell pacemaking. The test potential and the reference from which each value has been deduced are also shown.

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<th>Density (pA/pF)</th>
<th>Test potential (mV)</th>
<th>Reference</th>
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<td>$I_{Na}$ (TTX resistant)</td>
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<td>-20</td>
<td>11</td>
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<tr>
<td>$I_{Nas}$ (TTX sensitive)</td>
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<tr>
<td>$I_{Ca,L}$ (Ca,1.2)</td>
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<td>3</td>
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<tr>
<td>$I_{Ca,LD}$ (Ca,1.3)</td>
<td>10.4</td>
<td>-20</td>
<td>This study, see also 3</td>
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<td>$I_{f}$</td>
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<td>$I_{to}$</td>
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<td>$I_{sus}$</td>
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Supplementary table 3.

Model initial values. Abbreviations are defined as in the original model by Zhang et al. $d_{LD}$, $f_{LD}$, $d_{st}$, $f_{st}$ are the activation and inactivation gating variables for the Ca,1.3 and $I_{st}$ current, respectively.

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<td>$ms$</td>
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<td>$f_{LD}$</td>
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Mangoni et al. Supplementary Figure 1
Supplementary Figure 2

A

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<td>+/+</td>
<td>+/+</td>
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<tr>
<td>Hipp.</td>
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<td></td>
<td></td>
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<tr>
<td>-/-</td>
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B

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</tbody>
</table>

Mangoni et al.
Mangoni et al. Supplementary Figure 3
A

**ICaT (Cav3.1)**

$H_p = -90 \text{ mV}$

**ICaL (Cav1.3)**

$H_p = -60 \text{ mV}$

**Is**

$H_p = -80 \text{ mV}$

**INaTTXs**

$H_p = -120 \text{ mV}$

**IKr**

$H_p = -60 \text{ mV}$

**IK1 + If**

$H_p = -35 \text{ mV}$

B

**fT**

**fL**

**dT**

**dL**

**fLD**

**dLD**

C

**hS1**

**hL**

**m**

**ms**

*Mangoni et al. Supplementary Figure 4*
Supplementary Figure 5

(A) Voltage (mV) plot showing the effect of no ICa,T compared to control (Cont).

(B) Current (nA) plot illustrating various currents including iKr, iNaTX, iCa (Cav1.2), iCa (Cav1.3), and iCl (Cav3.1).

(C) Expanded view of currents showing different components and their contributions.

(D) Voltage (mV) vs. current (nA) plot highlighting the voltage range and corresponding currents.

(E) Expanded view of current components showing specific voltages and currents.

(F) Voltage (mV) plot similar to (A) but with a different time scale and labels indicating the effect of no ICa,T.

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