Online data supplements to be published at the Circulation Research web site:

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 \( \text{Ca}_{v3.1}^{-/-} \) adult mice as previously described \(^1\). 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. \( \text{Ca}^{2+} \) currents were recorded at 26°C with the conventional whole-cell patch-clamp technique using extracellular 2 mM \( \text{Ca}^{2+} \) and analyzed as described previously \(^3\). The cell access resistance in these recording conditions was around 10 M\( \Omega \), series resistance was evaluated at the peak of \( I_{Ca} \) and could be corrected up to 75%. Capacitative transients were not compensated electronically. \( \text{Ca}^{2+} \) currents were recorded using an extracellular solution containing (in mmol/L): TEA-Cl, 130; \( \text{CaCl}_2 \), 2; \( \text{MgCl}_2 \), 1; 4-amino-pyridine, 10; HEPES, 25; (adjusted to pH 7.4 with TEAOH). Whole-cell pipettes were filled with a solution containing (mM) \( \text{CsCl} \), 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 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-Mg²⁺, 0.1; CaCl₂, 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; CaCl₂, 1.8; MgCl₂, 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. 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. 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. 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 Ca,3.1±/− 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 Ca,3.1±/− 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 \(\text{Ca}_{v}3.1\), \(\text{Ca}_{v}3.2\) and \(\text{Ca}_{v}3.3\) were amplified using the following set of primers:
\(\text{Ca}_{v}3.1\)-F 5'-GTCTCCGCACGGTCTGTAAC-3' (NM_009783, nucleotide (nt) 329), \(\text{Ca}_{v}3.1\)-R,
5'-CCACAGCAAAGAA GGCAAAG-3' (nt 499, expected size 170bp), \(\text{Ca}_{v}3.2\)-F 5'-TGGGAACGTGCTTTCTTCTCT-3'
(NM_021415, nt 690), \(\text{Ca}_{v}3.2\)-R,
GGGGATGTGTGAGCATTTCT (nt 918, expected size 228bp), Ca\textsubscript{v}3.3-F 5’-GACACCACTCCCCACACACT-3’(XM\_139476, nt 3250), Ca\textsubscript{v}3.3-R, CCTCCTGTTTCTCCTCCTTC-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 MgCl\textsubscript{2} 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 Ca\textsubscript{v}3.1, 55°C for Ca\textsubscript{v}3.2 and 59°C for Ca\textsubscript{v}3.3. 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**

Ca\textsubscript{v}3 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 Ca\textsubscript{v}3.1, Ca\textsubscript{v}3.2 and Ca\textsubscript{v}3.3 and hypoxanthine guanine phosphoribosyl transferase (HPRT) : Ca\textsubscript{v}3.1 : Q-Ca\textsubscript{v}3.1-F 5’-CCAAGCAACGGGAGAGTCA-3’ (NM\_009783, nt 1310) and Q-Ca\textsubscript{v}3.1-R 5’-GCTAGCATTGGACAGGAATCG-3’ (nt 1371, expected size 61bp) ; Ca\textsubscript{v}3.2 : Q- Ca\textsubscript{v}3.2-F 5’-CAGCGGCTACGCCTATGC-3’(NM\_021415, nt 3230) and Q-Ca\textsubscript{v}3.2-R 5’-CAAAAGAGTGTGGGCCATGTC-3’(nt 3294, expected size 65bp) ; Ca\textsubscript{v}3.3 : Ca\textsubscript{v}3.3-F 5’-CCTTTTTTGCTCTTGTGTTTTTCTG-3’ (XM\_139476 nt 1106), Ca\textsubscript{v}3.3-R 3’-ACTTTAGGGAGGACATCATATTATTCC-3’ (nt 1219, expected size 113bp) and HPRT : Q-HPRT-F 5’-GCAGTACAGCCCCAAAATGG-3’ (NM\_013556, nt 540), and Q-HPRT-R 5’-GGTCCTTTTACCAGCAAGCT-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\(_v\)1.3- and Ca\(_v\)1.2- mediated \(I_{Ca,L}\), according to previous studies on Ca\(_v\)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\(_v\)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 (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 \( I_{\text{Ca,T}} \)**

A Hodgkin-Huxley (HH) model of \( \text{Ca}v3.1 \)-mediated \( I_{\text{Ca,T}} \) was constructed according to the equations described in Destexhe & Huguenard. Steady state parameters for activation were taken from our \( I_{\text{Ca,T}} \) records in SAN cells from WT mice. Steady-state inactivation parameters of \( I_{\text{Ca,T}} \) were from our previous study on SAN cells from \( \text{Ca}v1.3^{-/-} \) mice which allow precise measurement of \( I_{\text{Ca,T}} \) 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 \( I_{\text{Ca,T}} \) from \( \text{Ca}v1.3^{-/-} \) mice. For \( I_{\text{Ca,T}} \) reactivation kinetics, we used experimental records obtained from recombinant \( \text{Ca}v3.1b \) channels which are expressed in the heart and show the greatest similarity in steady state properties with the native mouse SAN \( I_{\text{Ca,T}} \).

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

For modelling of \( I_{\text{Ca,L}} \), we have used the same equations as in Zhang et al. According to experimental evidence from SAN cells of \( \text{Ca}v1.3^{-/-} \) mice, we have assumed that the total whole-cell \( I_{\text{Ca,L}} \) is composed by both \( \text{Ca}v1.3 \) and \( \text{Ca}v1.2 \) channels. Two independent groups of equations were used for \( \text{Ca}v1.3 \)- and \( \text{Ca}v1.2 \)-mediated \( I_{\text{Ca,L}} \). Activation and inactivation steady-state parameters used in the model, as well as current densities of \( \text{Ca}v1.3 \)- and \( \text{Ca}v1.2 \)-
mediated $I_{Ca,L}$ are from our previous study on Ca$_v$1.3-/- 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 ($I_{Na,s}$) and TTX-resistant ($I_{Na,r}$) $I_{Na}$ according to Lei et al. Steady-state activation/inactivation parameters as well as the relative current densities of $I_{Na,s}$ and $I_{Na,r}$ 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. 

**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{v1.3} and Ca\textsubscript{v1.2}-mediated \(I_{Ca,L}\), \(^{3,10}\) as well as TTX-sensitive and TTX-resistant \(I_{Na}\) \(^{11}\). The model also includes \(I_{st}\) current according to a recent study on mouse SAN cells by Cho and coworkers \(^{12}\). \(I_{Kr}\) was also modelled to account for experimental data in Clark et al \(^{21}\). \(I_{K1}\) was also included in the model, since this current has been consistently found in mouse SAN cells \(^{12}\) (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{v1.3}-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{v3.1} and Ca\textsubscript{v1.3}-mediated currents since Ca\textsubscript{v1.3} channels are already partially inactivated at a Hp of -55 mV \(^{3}\) (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{v1.3} and Ca\textsubscript{v1.2}-mediated \(I_{Ca,L}\) \(^{3}\). As to TTX-sensitive and TTX-resistant \(I_{Na}\), our modelling work reproduced the data by Lei and co-workers \(^{11}\). 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. \(^{11}\)). The density of TTX-resistant \(I_{Na}\) was set as for cells having capacitance of 30 pF in Lei et al. \(^{11}\). 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,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), $I_{sf}$ 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,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,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,3.1-related $I_{Ca,T}$ indicate that TTX-sensitive $I_{Na}$ cannot compensate for the lack of $I_{Ca,T}$ in Ca,3.1−/− mice, at least under basal conditions. Ca,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,1.3 channels and/or to the predicted window Ca,1.3-mediated $I_{Ca,L}$ (supplementary Figure 4B). Incomplete inactivation of Ca,1.3-mediated $I_{Ca,L}$ has also been observed upon application of pacemaker action potential waveforms to HEK cells expressing recombinant human Ca,1.3 channels. This persistent $I_{Ca,L}$ component
contribute to the diastolic depolarisation by summing to Ca\textsubscript{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\textsubscript{v1.3}-related $I_{Ca,L}$ is the major voltage-dependent Ca\textsuperscript{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\textsubscript{v1.3}-related $I_{Ca,L}$, $I_{st}$ is strongly sensitive to $\beta$-adrenergic agonists\textsuperscript{12}. These currents can thus constitute two major mechanisms of compensation of the lack of Ca\textsubscript{v3.1} channels at maximal heart rates which is presumably driven by strong $\beta$-adrenergic input on the SAN (see table1). Compared to Ca\textsubscript{v1.3} and TTX-sensitive channels, Ca\textsubscript{v3.1} channels are strongly inactivated at positive MDPs. We can expect that the relative contribution of Ca\textsubscript{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 $\beta$-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\textsubscript{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\textsubscript{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,3.1-mediated $I_{Ca,T}$ 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 $I_{Ca,T}$ during pacemaker activity with respect to other ionic currents involved in the genesis and regulation of automaticity. Calculations indicate that Ca,3.1-mediated $I_{Ca,T}$ can contribute to the diastolic depolarisation by activating early in the diastolic depolarisation phase together with $I_{st}$. The contribution of Ca,3.1-mediated $I_{Ca,T}$ to overall pacemaking is predicted to be moderate, an observation which is experimentally verified. Our calculations are thus also important for indicating how Ca,3.1 channels can contribute to the diastolic depolarisation in spite being partly inactivated at positive MDPs. The physiological significance of Ca,3.1-related $I_{Ca,T}$ can also be linked to the presence of large deactivating $I_{Kr}$ at the end of repolarization. Indeed, the presence of $I_{Ca,T}$ 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,3.1-related $I_{Ca,T}$ (supplementary Figure 5A). Consistently with the work of Lei et al.¹¹, abolition of TTX-sensitive and resistant $I_{Na}$ affected the SAN rate, as well as the takeoff potential and the upstroke phase in our simulations (not shown). These observation indicate that Ca,3.1-related $I_{Ca,T}$ and $I_{Na}$ 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 Ca²⁺ concentration remains constant and to the available data on mouse SAN automaticity. Indeed, even if detailed modelling of diastolic release of Ca²⁺ have been developed for rabbit SAN cells²³, no quantitative experimental data are available on this newly described pacemaker mechanism in mouse SAN cells. Consequently, inclusion of RyR-dependent Ca²⁺ 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:

15. Chemin J, Monteil A, Bourinet E, Nargeot J, Lory P. Alternatively spliced \alpha_{1G} (CaV3.1) intracellular loops promote specific T-type Ca^{2+} channel gating properties. *Biophys J.* 2001;80:1238-1250.


Online Supplementary Figure Legends

Online supplementary Figure 1:

No difference in capacitance and cell morphology between WT and Ca_{3.1}^{-/-} cardiac cells. Electrophysiological recordings of SAN cells from WT and Ca_{3.1}^{-/-} 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 Ca_{3.1}^{-/-} 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 Ca_{3.1} 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 Ca^{2+} channel isotype mRNAs in wild-type (+/+ ) and Ca_{3.1}^{-/-} (-/-) mouse heart. Transcripts for the Ca_{3.1} and Ca_{3.2} subunits, but not for the Ca_{3.3} 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 Cav3.1 gene was assessed by using a set of primers overlapping from the first to the third exon. Expectedly, no Ca_{3.1} transcripts were detectable in Ca_{3.1}^{-/-} mice since the forward primer used to identify Ca_{3.1} transcripts was comprised within exon 1 that was deleted to generate a null allele for cacna1g. Because RT-PCR experiments indicated that Ca_{3.1} 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 $2^{-\Delta \text{Ct}} \times 100$. 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 $I_{\text{Ca,T}}$ 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 $I_{\text{Ca,T}}$ 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 Ca\textsubscript{v}3.1 (\(d_{T} f_{T}\), Ca\textsubscript{v}1.3 (\(d_{LD} f_{LD}\)) and Ca\textsubscript{v}1.2 (\(d_{L} f_{L}\)) 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 Ca\textsubscript{v}3.1-mediated \(I_{Ca,T}\) on a simulated SAN action potential obtained with parameters indicated in supplementary tables 1-3. (B). Activation of Ca\textsubscript{v}3.1-mediated \(I_{Ca,T}\) compared with Ca\textsubscript{v}1.3, Ca\textsubscript{v}1.2 and TTX-sensitive and resistant \(I_{Na}\) as indicated in the panel legend. (C). Activation during the pacemaker cycle of voltage-dependent K\textsuperscript{+} currents, together with \(I_{K1}\) and \(I_{f}\). (D). Current-to-voltage relationship of Ca\textsubscript{v}3.1, Ca\textsubscript{v}1.3 and Ca\textsubscript{v}1.2 channels during the pacemaker cycle shown in (A). The predicted \(I_{s}\) current is also shown here. (E). Current to voltage relationships comparing Ca\textsubscript{v}3.1 channels to TTX-sensitive and TTX-resistant \(I_{Na}\) as indicated in the panel legend, for the pacemaker cycle in (A). (F). Effects of Ca\textsubscript{v}3.1 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 µS.
**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.\(^9\). Other abbreviations define the conductance of \(I_{st} (g_{st})\) and that of \(I_{KI} (g_{KI})\).

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Absolute value</th>
<th>Abbreviation</th>
<th>Absolute value</th>
</tr>
</thead>
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<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>
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<td>(E_{Ca,L})</td>
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<tr>
<td></td>
<td>45 mV</td>
<td>(g_{sus})</td>
<td>0.000065 µS</td>
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<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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<td>(I_{Na} V_{0.5act})</td>
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<td>(g_{I_{Na}})</td>
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<td>(g_{I_{K}})</td>
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<td>(G_{Nas} (TTX sensitive))</td>
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<td>(\gamma_{NaCa})</td>
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<tr>
<td>(I_{Ca,D} V_{0.5act} (Ca_{1.3}))</td>
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<td>(k_{NaCa})</td>
<td>0.000027 nA</td>
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<td>(I_{Ca,D} V_{0.5inact} (Ca_{1.3}))</td>
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<td>([Na^+]_o)</td>
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<tr>
<td>(g_{Ca,T})</td>
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<td>([Na^+]_i)</td>
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<td>(g_{st})</td>
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<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>-20</td>
<td>11</td>
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<tr>
<td>$I_{NaS}$ (TTX sensitive)</td>
<td>135</td>
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<td>$I_{Ca,L}$ $(Ca_{1.2})$</td>
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<td>+10</td>
<td>3</td>
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<td>$I_{Ca,L,D}$ $(Ca_{1.3})$</td>
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<td>-20</td>
<td>This study, see also 3</td>
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<td>$I_{Ca,T}$</td>
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<td>This study</td>
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<td>$I_{st}$</td>
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<td>9</td>
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Supplementary table 3.

Model initial values. Abbreviations are defined as in the original model by Zhang et al 9. $d_{LD}$, $f_{LD}$, $d_{st}$, $f_{st}$ are the activation and inactivation gating variables for the $Ca_{1.3}$ and $I_{st}$ current, respectively.

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<th>$V_M$ (mV)</th>
<th>$m$</th>
<th>$h_1$</th>
<th>$h_2$</th>
<th>$m_s$</th>
<th>$h_{S1}$</th>
<th>$h_{S2}$</th>
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<th>$f_L$</th>
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<table>
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<th>$r$</th>
<th>$q$</th>
<th>$n$</th>
<th>$p_{b,f}$</th>
<th>$p_{b,s}$</th>
<th>$p_i$</th>
</tr>
</thead>
</table>
Mangoni et al. Supplementary Figure 1
Mangoni et al. Supplementary Figure 2
Mangoni et al. Supplementary Figure 3
\[ I_{CaT} \quad (Cav3.1) \quad H_p = -90 \text{ mV} \]
\[ I_{CaL} \quad (Cav1.3) \quad H_p = -60 \text{ mV} \]
\[ I_{s} \quad H_p = -80 \text{ mV} \]

\[ I_{NaTTXs} \quad H_p = -120 \text{ mV} \]
\[ I_{Kr} \quad H_p = -60 \text{ mV} \]
\[ I_{K1} + I_{f} \quad H_p = -35 \text{ mV} \]

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