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Functional roles of $Ca_v1.3$, $Ca_v3.1$ and HCN channels in automaticity of mouse atrioventricular cells

Insights into the atrioventricular pacemaker mechanism

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Key words: genetically-engineered mice, pacemaker activity, atrioventricular node, congenital heart block, sino-atrial node dysfunction, ion channels, $Ca_v1.3$ channels, $Ca_v3.1$ channels, HCN channels, electrophysiology, conduction, heart rate

Abberviations: AVN, atrioventricular node; SAN, sino-atrial node; $I_{Ca,L}$, L-type Ca^{2+} current; $I_{Ca,T}$, T-type Ca^{2+} current; I_f, hyperpolarization-activated f-current; $Ca_v1.3$, voltage-dependent L-type Ca^{2+} channel 3rd isoform; $Ca_v3.1$, voltage-dependent T-type Ca^{2+} channel 1st isoform; HCN, hyperpolarization-activated cation channel; CHB, congenital heart block; SNDD, sino-atrial dysfunction and deafness syndrome

The atrioventricular node controls cardiac impulse conduction and generates pacemaker activity in case of failure of the sino-atrial node. Understanding the mechanisms of atrioventricular automaticity is important for managing human pathologies of heart rate and conduction. However, the physiology of atrioventricular automaticity is still poorly understood. We have investigated the role of three key ion channel-mediated pacemaker mechanisms namely, $Ca_v1.3$, $Ca_v3.1$ and HCN channels in automaticity of atrioventricular node cells (AVNCs). We studied atrioventricular conduction and pacemaking of AVNCs in wild-type mice and mice lacking $Ca_v3.1$ ($Ca_v3.1^{-/-}$), $Ca_v1.3$ ($Ca_v1.3^{-/-}$), channels or both ($Ca_v1.3^{-/-}$/$Ca_v3.1^{-/-}$). The role of HCN channels in the modulation of atrioventricular cells pacemaking was studied by conditional expression of dominant-negative HCN4 channels lacking cAMP sensitivity. Inactivation of $Ca_v3.1$ channels impaired AVNCs pacemaker activity by favoring sporadic block of automaticity leading to cellular arrhythmia. Furthermore, $Ca_v3.1$ channels were critical for AVNCs to reach high pacemaking rates under isoproterenol. Unexpectedly, $Ca_v1.3$ channels were required for spontaneous automaticity, because $Ca_v1.3^{-/-}$ and $Ca_v1.3^{-/-}$/$Ca_v3.1^{-/-}$ AVNCs were completely silent under physiological conditions. Abolition of the cAMP sensitivity of HCN channels reduced automaticity under basal conditions, but maximal rates of AVNCs could be restored to that of control mice by isoproterenol. In conclusion, while $Ca_v1.3$ channels are required for automaticity, $Ca_v3.1$ channels are important for maximal pacing rates of mouse AVNCs. HCN channels are important for basal AVNCs automaticity but do not appear to be determinant for β-adrenergic regulation.

Introduction

The atrioventricular node (AVN) sets the proper delay between atrial and ventricular activation, and can drive the heartbeat in case of failure of the sino-atrial node (SAN).[1] The AVN is also an important pharmacological target for controlling ventricular rate upon atrial arrhythmias. One of the most fascinating features of AVN tissue is the coexistence of a highly regulated conduction function and automaticity. Interestingly, SAN and AVN dysfunction are often found in association in human pathologies of heart rhythm. For instance, sick sinus syndrome caused by genetic mutations in the *Scn5A* gene is characterized by SAN bradycardia and dysfunction of atrioventricular (AV) conduction.[2,3] In congenital heart block (CHB) SAN bradycardia is associated with different degrees of AV conduction dysfunction.[4] It has been proposed that CHB is caused by circulating maternal autoantibodies against L-type $Ca_v1.3$ and possibly T-type $Ca_v3.1$ channels in plasma of affected children.[5,6] In the recently described "sinus node dysfunction and deafness syndrome" (SNDD), SAN bradycardia is associated to atrioventricular blocks and rhythm

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dissociation.[7] SNDD is caused by insertion of a glycine residue into a highly conserved alternatively spliced region of the $Ca_v1.3$ pore-forming subunit, a condition that produces non-conducting $Ca_v1.3$ channels.[7] Specific phenotypes of genetically-modified mouse strains in which altered genes coding for ion channels involved in cardiac automaticity have been described (reviewed in ref. 8), among them, mice lacking $Ca_v1.3$ channels,[9,10] $Ca_v3.1$ channels,[11] or *Scn5A* haplo-insufficient mice (*Scn5A*$^{+/-}$),[12] with reduced cardiac TTX-resistant Na^+ current ($I_{Na,r}$) and mice lacking Ca^{2+} activated SK2 K^+ channels[13] show dysfunction in both SAN pacemaker activity and impulse conduction through the AVN. Association between SAN and AVN dysfunction in mouse models indicates that genetically-modified strains are a promising approach towards understanding the bases of AVN automaticity and AV conduction, but we have limited information of the functional role of ion channel isoforms in spontaneously active mouse AVN cells (AVNCs). Better knowledge of AVN automaticity is thus important for understanding human pathologies of heart rhythm and also for developing new therapies which aim to stimulate AVN pacemaking with concomitantly improved ventricular rate control. Since genetic loss of $Ca_v1.3$ and $Ca_v3.1$ channels affects both SAN automaticity and AV conduction,[9-11] the primary objective of the present study was to investigate the functional roles of these channels in AVNCs pacemaking. Furthermore, because cAMP-dependent regulation of hyperpolarization-activated HCN (f-) channels is considered to play a key role in autonomic control of SAN pacemaking[14] and maximal heart rates,[15] we aimed to compare the effects of genetic modification of $Ca_v1.3$, $Ca_v3.1$ and HCN channels on the β-adrenergic regulation on AVNC automaticity. Interestingly, we found that these channels differentially impact AVNC automaticity. Furthermore, inactivation of $Ca_v1.3$ channels prevents spontaneous pacemaking of AVNCs, a finding that can explain the pathophysiologic bases of SNDD. We also report that combined loss of $Ca_v1.3$ and $Ca_v3.1$ channels induces SAN and AVN dysfunction in mice similar to human CHB. Our work establishes the role of $Ca_v1.3$, $Ca_v3.1$ and HCN channels in AVNCs pacemaking and gives insights into the pathogenesis of dysfunction of human AV conduction such as CHB and SNDD.

Results

Atrioventricular dysfunction in mice lacking Ca_v channels. We first evaluated heart rate and AV conduction dysfunction in mice lacking $Ca_v1.3$, $Ca_v3.1$ channels or both in telemetric recordings in freely moving animals. $Ca_v3.1^{-/-}$ mice showed regular heartbeats with moderately reduced heart rates and longer PR intervals than wild-type (WT) mice, consistently to what has been reported previously in animals with a different genetic background (**Fig. 1A and B and Sup. Table S1**).[11] $Ca_v1.3^{-/-}$ mice showed irregular heart rate with intermittent AV blocks. PR intervals of $Ca_v1.3^{-/-}$ mice were longer than that of $Ca_v3.1^{-/-}$ mice (**Fig. 1A and B**). To determine if concomitant loss of $Ca_v1.3$ and $Ca_v3.1$ channels resulted in an additive effect on AV conduction we generated $Ca_v1.3^{-/-}$/$Ca_v3.1^{-/-}$ mice by crossing $Ca_v1.3^{-/-}$ with $Ca_v3.1^{-/-}$ mice. $Ca_v1.3^{-/-}$/$Ca_v3.1^{-/-}$ mice showed severely delayed AV conduction, with longer PR intervals compared to single mutants for each channel (**Fig. 1A and B**). Complete AV block and rhythm dissociation were also observed (**Fig. 1A**). Furthermore, more frequent episodes of high degree AV block were observed in $Ca_v1.3^{-/-}$/$Ca_v3.1^{-/-}$ mice compared to each single mutant strain. These episodes were characterized by the presence of multiple isolated P waves that were not followed by a QRS complex. Thus, the averaged number of isolated P waves was higher in $Ca_v1.3^{-/-}$/$Ca_v3.1^{-/-}$ mice than in $Ca_v1.3^{-/-}$ mice (**Fig. 1C and Sup. Table S1**). The mean heart rate was increasingly slower in $Ca_v3.1^{-/-}$, $Ca_v1.3^{-/-}$ and $Ca_v1.3^{-/-}$/$Ca_v3.1^{-/-}$ mice. These observations showed that combined loss of $Ca_v1.3$ and $Ca_v3.1$ channels produced a higher impact on heart rate and AV conduction than the $Ca_v1.3^{-/-}$ mutation alone.

$Ca_v1.3$ channels are required for mouse AVNC automaticity. Previous studies have shown that $Ca_v1.3$-mediated $I_{Ca,L}$ is important for pacemaking of SANCs.[10,16] The dependence of WT AVNC automaticity from $I_{Ca,L}$ (see the accompanying paper in ref. 17) and the dysfunction of AV conduction observed in $Ca_v1.3^{-/-}$ mice suggest that $Ca_v1.3$ channels are functionally important in AVNCs. We thus speculated that $Ca_v1.3$ channels could play a role in automaticity of AVNCs and tested the contribution of $Ca_v1.3$-mediated $I_{Ca,L}$ to AVNC pacemaking using $Ca_v1.3^{-/-}$ mice. Inactivation of $Ca_v1.3$-mediated $I_{Ca,L}$ had severe effects on AVNC automaticity. All tested $Ca_v1.3^{-/-}$ AVNCs did not show spontaneous pacemaking, but displayed a depolarized membrane potential of -32 ± 4 mV (**Fig. 2A** and n = 11). This membrane potential did not significantly differ from that observed in WT cells treated with isradipine ($p > 0.05$).[17] Also similar to isradipine treated WT AVNCs, $Ca_v1.3^{-/-}$ AVNCs displayed low amplitude oscillations of membrane potential (**Fig. 2A**). The membrane voltage oscillations recorded in isradipine treated AVNCs were thus unlikely to be caused by unblocked $Ca_v1.3$ channels.[17]

Both submaximal (0.02 μM) and saturating (0.1 μM) concentrations of isoproterenol (ISO) could not restore spontaneous pacemaking of $Ca_v1.3^{-/-}$ AVNCs (data not shown).

To test the possibility that the lack of automaticity in $Ca_v1.3^{-/-}$ AVNCs was due to failure to generate the AP upstroke, we hyperpolarized $Ca_v1.3^{-/-}$ AVNCs by injection of a constant negative current (-25 ± 2 pA, n = 11) until the cell fired APs spontaneously (**Fig. 2D**). Out of eleven cells tested, seven $Ca_v1.3^{-/-}$ AVNCs showed spontaneous APs upon current injection, while four cells (36% of total) remained silent. Because $Ca_v1.3^{-/-}$ cells could fire APs only upon tonic negative current injection, we will refer to their activity as "initiated automaticity" rather than "pacemaker activity". $Ca_v1.3^{-/-}$ AVNCs exhibited initiated automaticity from a MDP of -60 ± 2 mV n = 7, a value that did not differ from the MDP of WT AVNCs (see Table 1 of the accompanying paper ref. 17). However, initiated automaticity of negative current-injected $Ca_v1.3^{-/-}$ AVNCs was slower than pacemaker activity of WT cells (**Sup. Table S2**). Furthermore, while pacemaking of WT AVNCs was regular and presented linear diastolic depolarization, initiated automaticity of negative current-injected $Ca_v1.3^{-/-}$ AVNCs was intermittent and did not present a depolarizing diastolic phase (**Fig. 2B**). Instead rather than a diastolic depolarization phase, $Ca_v1.3^{-/-}$ AVNCs presented subthreshold oscillations of the membrane potential similar to

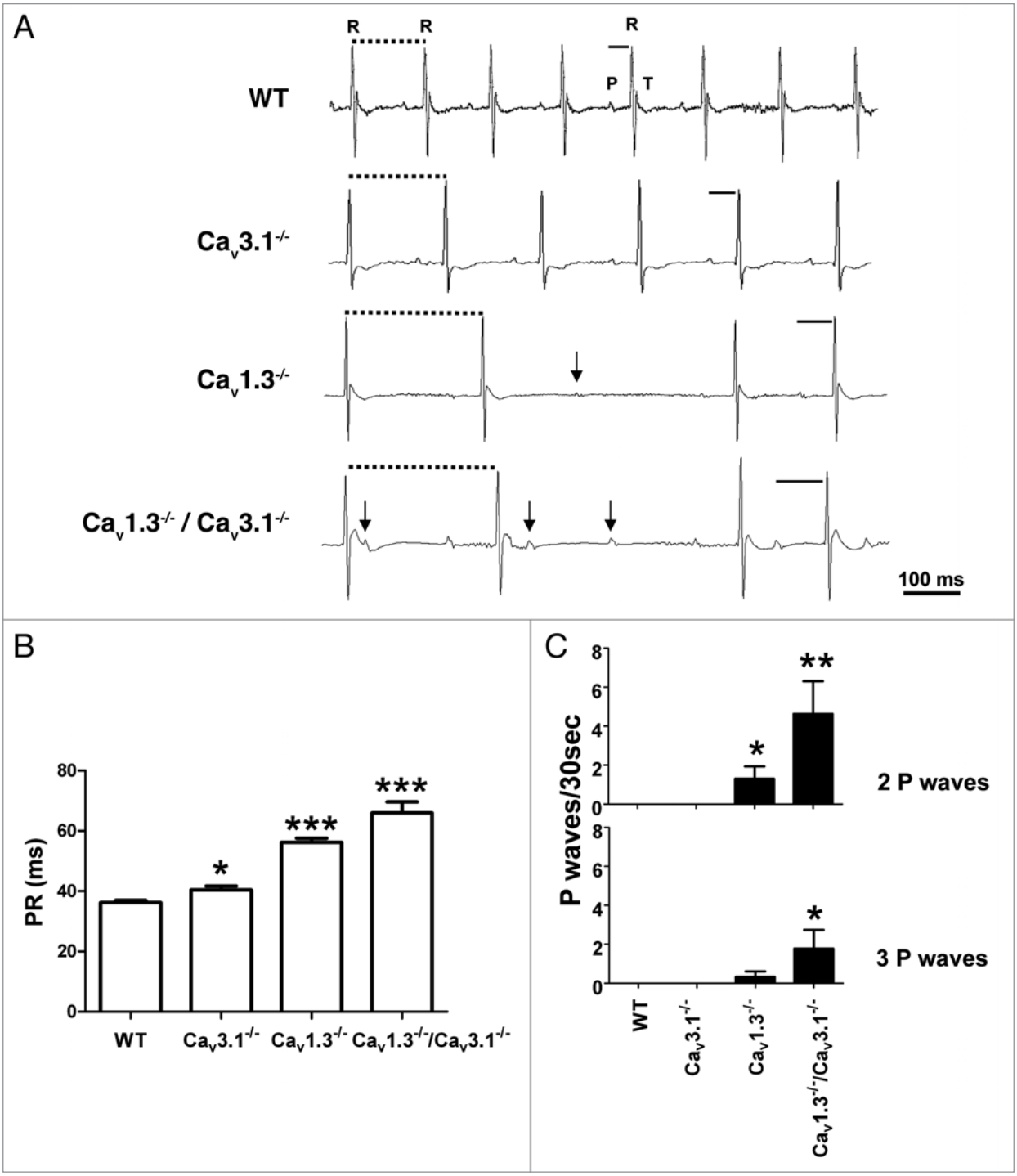

Figure 1. (A) Telemetric surface ECGs of freely moving WT, $Ca_v3.1^{-/-}$, $Ca_v1.3^{-/-}$ and $Ca_v1.3^{-/-}/Ca_v3.1^{-/-}$ mice showed additive effect of Ca_v gene inactivation on AV conduction dysfunction. Solid bars indicate PR interval duration in different genotypes. Dotted bars indicate RR intervals. Arrows indicate isolated P waves. Note that in $Ca_v1.3^{-/-}/Ca_v3.1^{-/-}$ mice, some P waves elicited a QRS complex and some isolated P waves fall into the T wave of the QRS complex. PR intervals were progressively longer in $Ca_v3.1^{-/-}$, $Ca_v1.3^{-/-}$ and $Ca_v1.3^{-/-}/Ca_v3.1^{-/-}$ mice. (B) Stars indicate statistical significance compared to WT (one-way ANOVA). (C) Isolated P waves were not found in WT and $Ca_v3.1^{-/-}$ mice. The number of two or three consecutive isolated P waves measured was higher in $Ca_v1.3^{-/-}/Ca_v3.1^{-/-}$ than in $Ca_v1.3^{-/-}$ mice, indicating that AV conduction dysfunction in a $Ca_v1.3^{-/-}$ genetic background was worsened by additional loss of $Ca_v3.1$ channels. Stars indicate statistical significance compared to WT (Kruskal-Wallis).

those seen after isradipine block of WT APs.[17] These randomly reached the threshold for AP discharge (**Fig. 2B**). The AP amplitude was significantly lower in injected $Ca_v1.3^{-/-}$ AVNCs than in WT cells suggesting that $Ca_v1.3$ channels not only determine the diastolic depolarization but also contribute to the AP itself. The AP duration of negative current-injected $Ca_v1.3^{-/-}$ AVNCs tended

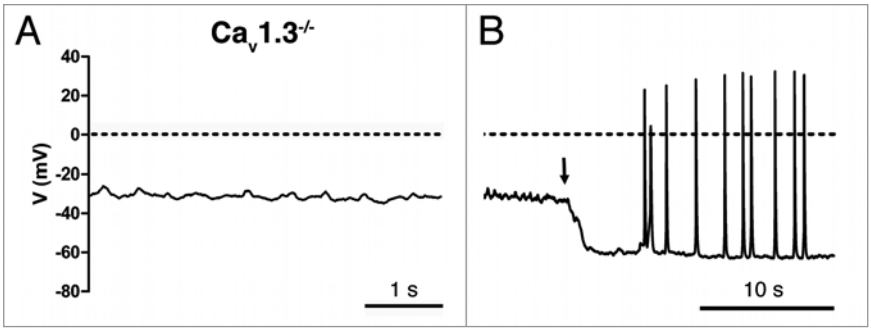

Figure 2. (A) $Ca_v1.3^{-/-}$ AVNCs display positive membrane potential and no basal pacemaker activity. (B) Tonic hyperpolarizing current injection (black arrow) induced spontaneous AP firing in $Ca_v1.3^{-/-}$ AVNCs.

to be longer than that of WT cells, but such a difference did not reach statistical significance (**Sup. Table S2**). Low residual (11% of the WT density) I_{Ca} was recorded in AVNCs of $Ca_v1.3^{-/-}$ mice (-2 ± 0.3 pA/pF n = 4 and -0.21 ± 0.03 pA/pF n = 6 in WT and $Ca_v1.3^{-/-}$ AVNCs, respectively). Most likely, $Ca_v1.2$ channels activating at higher voltages than $Ca_v1.3$ channels underlie this residual $I_{Ca,L}$ (**Fig. 3**). These observations indicate that $Ca_v1.3$ channels are required for pacemaking of mouse AVNCs and constitute the predominant L-type channel isoform in these cells.

$Ca_v3.1^{-/-}$ channels are important for AVNCs automaticity and determine maximal pacemaking rates in AVNCs. We reported in a previous study, that inactivation of $Ca_v3.1$ channels abolished $I_{Ca,T}$ in both SANCs and AVNCs.[11] The importance of $Ca_v3.1$-mediated $I_{Ca,T}$ in AVNCs automaticity is unknown. We thus investigated the impact of $Ca_v3.1$ channel inactivation in AVNC automaticity. Furthermore, because combined inhibition of $Ca_v1.3$ and $Ca_v3.1$ channel activity in CHB cause AV dysfunction, which is similar to that observed in $Ca_v1.3^{-/-}$/$Ca_v3.1^{-/-}$ mice (**Fig. 1**), we studied pacemaker activity in $Ca_v1.3^{-/-}$/$Ca_v3.1^{-/-}$ AVNCs.

Contrary to $Ca_v1.3^{-/-}$ cells, $Ca_v3.1^{-/-}$ AVNCs had spontaneous pacemaking (**Fig. 4**). However, $Ca_v3.1^{-/-}$ cells showed irregular and slower pacemaking than WT AVNCs (**Fig. 4A and B**). Similarly to $Ca_v1.3^{-/-}$ cells (**Fig. 4C**), $Ca_v1.3^{-/-}$/$Ca_v3.1^{-/-}$ AVNCs were quiescent at rest and injection of negative current (-23 ± 3 pA, n = 6) was necessary to initiate automaticity (**Fig. 4D**). $Ca_v3.1$ channel deficiency reduced AVNC pacemaking frequency by 70% compared to that of WT cells. Initiated automaticity of negative current-injected $Ca_v1.3^{-/-}$/$Ca_v3.1^{-/-}$ AVNCs was even slower than that of firing of negative current-injected $Ca_v1.3^{-/-}$ or pacemaker activity of $Ca_v3.1^{-/-}$ AVNCs. The firing rate of $Ca_v1.3^{-/-}$/$Ca_v3.1^{-/-}$ AVNCs was 86% slower than pacemaking of WT cells (**Fig. 4D and E and Sup. Table S2**). This observation indicates that $Ca_v3.1$-mediated $I_{Ca,T}$ does not only participate in the pacemaking of WT AVNCs, but contributes also to initiated automaticity of negative current-injected $Ca_v1.3^{-/-}$ AVNCs.

Because SAN and AVN activity are tightly controlled by β-adrenergic regulation, we also investigated how the loss of $Ca_v3.1$ and $Ca_v1.3$ channels affected the regulation of AVNC automaticity by ISO. In $Ca_v3.1^{-/-}$ AVNCs, 0.1 μM ISO significantly increased pacemaker activity, although maximal frequency was still lower than pacemaking of WT AVNCs under the same conditions (**Fig. 5A and B and Sup. Table S3**). Similarly, ISO stimulated initiated automaticity of negative current-injected $Ca_v1.3^{-/-}$ AVNCs but did not restore ISO-stimulated AP frequency measured in WT cells (**Fig. 5A and C and Sup. Table S4**). In both $Ca_v3.1^{-/-}$ and negative current-injected $Ca_v1.3^{-/-}$ AVNCs, the relative increase in pacemaking of $Ca_v3.1^{-/-}$ and initiated automaticity of negative current-injected AVNCs by ISO was similar to that of WT AVNCs (**Fig. 5D and E**). ISO did not normalize cellular arrhythmia of both $Ca_v3.1^{-/-}$ and negative current-injected $Ca_v1.3^{-/-}$ AVNCs (**Fig. 5B**).

cAMP regulation of HCN channels is important for basal AVNCs automaticity, but is not required to reach maximal pacemaking rates. In SANCs, HCN channels are considered to play a key role in both the generation and regulation of pacemaker activity, primarily because of the direct cAMP-dependent regulation of the channel open probability, which modulates I_f availability at voltages spanning that of the diastolic depolarization.[18] Because initiated automaticity of negative current-injected $Ca_v1.3^{-/-}$ and pacemaking of $Ca_v3.1^{-/-}$ AVNCs are still responsive to ISO (**Fig. 5D and E**) we investigated the importance of cAMP-dependent regulation of HCN-channels in AVNC pacemaking. For this purpose, we used the mutant mouse strain hHCN4-573X (see Materials and Methods). This strain expresses a dominant-negative cAMP-insensitive, HA-tagged human HCN4 channel construct in a heart-specific and inducible way.[15] Mutant mice expressing hHCN4-573X showed a negative shift of the I_f activation curve, which induces a functional loss of I_f activity at physiological diastolic voltages.[15]

AVNCs from control mice (**Fig. 6A** and right part) expressed HCN4 (see **Sup. Expanded Materials and Methods** and Fig. 1 in the accompanying paper in ref. 17). No specific anti-HA staining was observed in these cells (**Fig. 6A** and left part). In contrast, anti-HA staining was consistently found along membrane borders of mutant cells, showing that hHCN4-573X channels were targeted to the cell membrane (**Fig. 6B**). In perforated-patch voltage-clamp experiments, expression of hHCN4-573X in mutant AVNCs did not affect peak I_f density (**Fig. 6C–F**). Application of 0.1 μM ISO enhanced I_f in control cells (**Fig. 6D**), but failed to stimulate I_f in mutant AVNCs throughout the voltage range tested (**Fig. 6F**). Under current-clamp conditions, hHCN4-573X expressing AVNCs displayed irregular pacemaking (**Fig. 7A**). The AP amplitude of hHCN4-573X expressing cells was lower that that of control cells (**Sup. Table S4**). The membrane potential of hHCN4-573X expressing AVNCs was 9 mV more positive than that of control AVNCs. (**Sup. Table S4**). Application of a submaximal dose of ISO (0.02 μM) induced a small increase in pacemaking of control and mutant AVNCs (**Fig. 7B**). No significant rate difference between hHCN4-573X expressing cells and control AVNCs was observed at 0.1 μm ISO. At this ISO

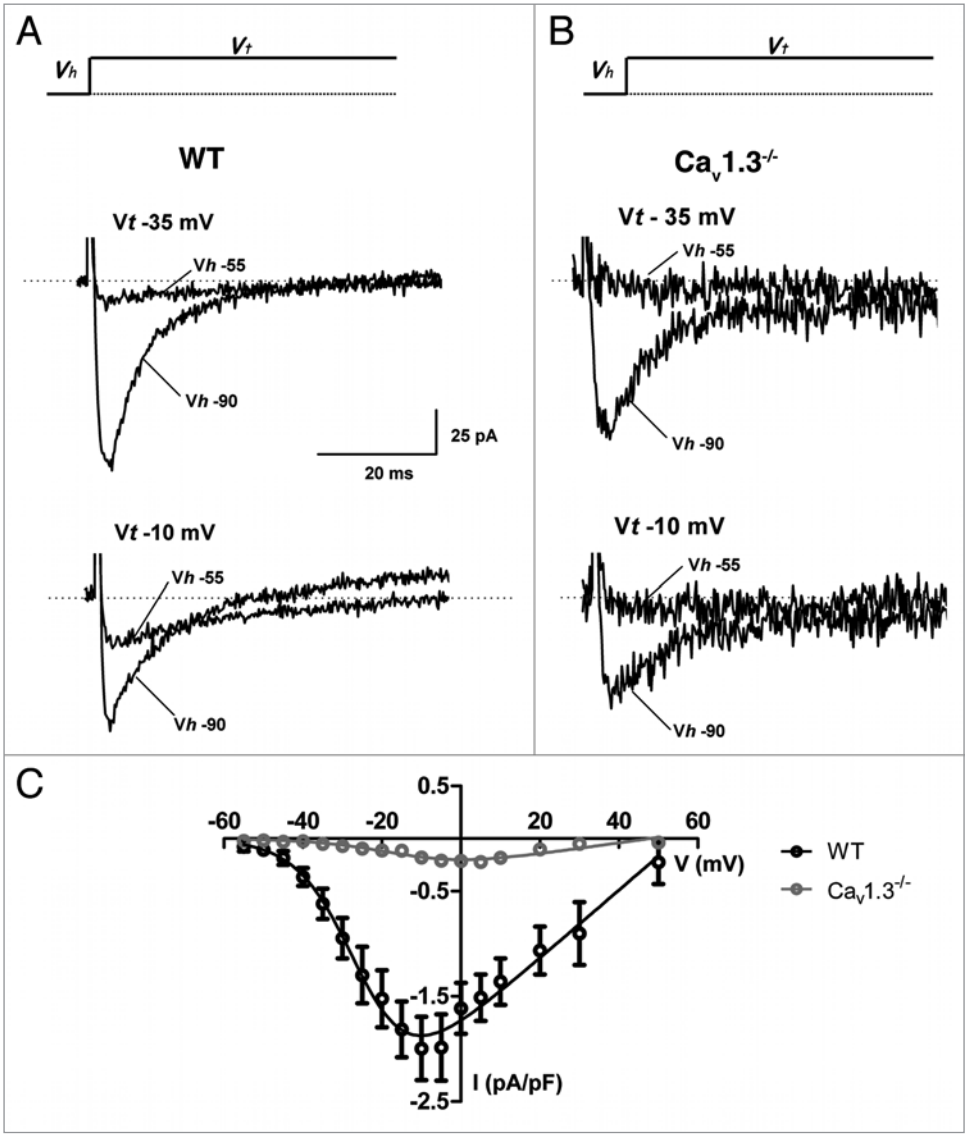

Figure 3. AVNCs of $Ca_v1.3^{-/-}$ mice display drastically reduced $I_{Ca,L}$. (A) Sample traces of $I_{Ca,L}$ in WT AVNCs at the two different test potentials (V_t) indicated (-35 and -10 mV). $I_{Ca,L}$ was activated from a holding potential (V_h) of -55 mV which inactivates $I_{Ca,T}$. (B) In contrast, very few $I_{Ca,L}$ is recorded in $Ca_v3.1^{-/-}$ AVNCs in the same condition. $I_{Ca,T}$ is activated from a HP of -90 mV in (A and B). (C) IV-curve of $I_{Ca,L}$ in WT (open black circles and line) and residual $I_{Ca,L}$ $Ca_v1.3^{-/-}$ mice (open red circles and line).

concentration, the AP amplitude and the MDP of hHCN4-573X expressing cells were indistinguishable from that of control AVNCs (**Sup. Table S4**).

Discussion

This is the first study investigating the functional role of three key ion channel genes in pacemaker activity of mouse AVNCs. Four major new findings are presented. First, $Ca_v1.3$ channels constitute the predominant $I_{Ca,L}$ channel isoform and are necessary for spontaneous automaticity of mouse AVNCs. In this respect, our results on $Ca_v1.3^{-/-}$ AVNCs give an important insight on the pathophysiologic mechanism of AV dysfunction in human SNDD. Second, we show that the loss of $Ca_v3.1$ channels restricts both basal and maximal spontaneous pacemaking rates of AVNCs. Third, we report that combined inactivation of $Ca_v1.3$ and $Ca_v3.1$ channels produces additive effects on both in vivo AV conduction and AVNCs firing and yields a phenotype similar to human CHB. Forth, we found that cAMP-dependent regulation of HCN channels appears to be important for AVNC automaticity under basal conditions, but is not strictly required for regulation of pacemaking and for reaching maximal pacing rates, especially under β-adrenergic receptor activation. Our study thus provides a comprehensive description of the pacemaker activity of murine AVNCs.

$Ca_v1.3$ channels are required for AVNC pacemaking. The loss of $Ca_v1.3$ channels induces AV conduction dysfunction in mice (**Fig. 1**), suggesting that these channels play an important role in AVN physiology. In this study, we show that $Ca_v1.3$ channels play an essential role in AVNCs pacemaking. Several

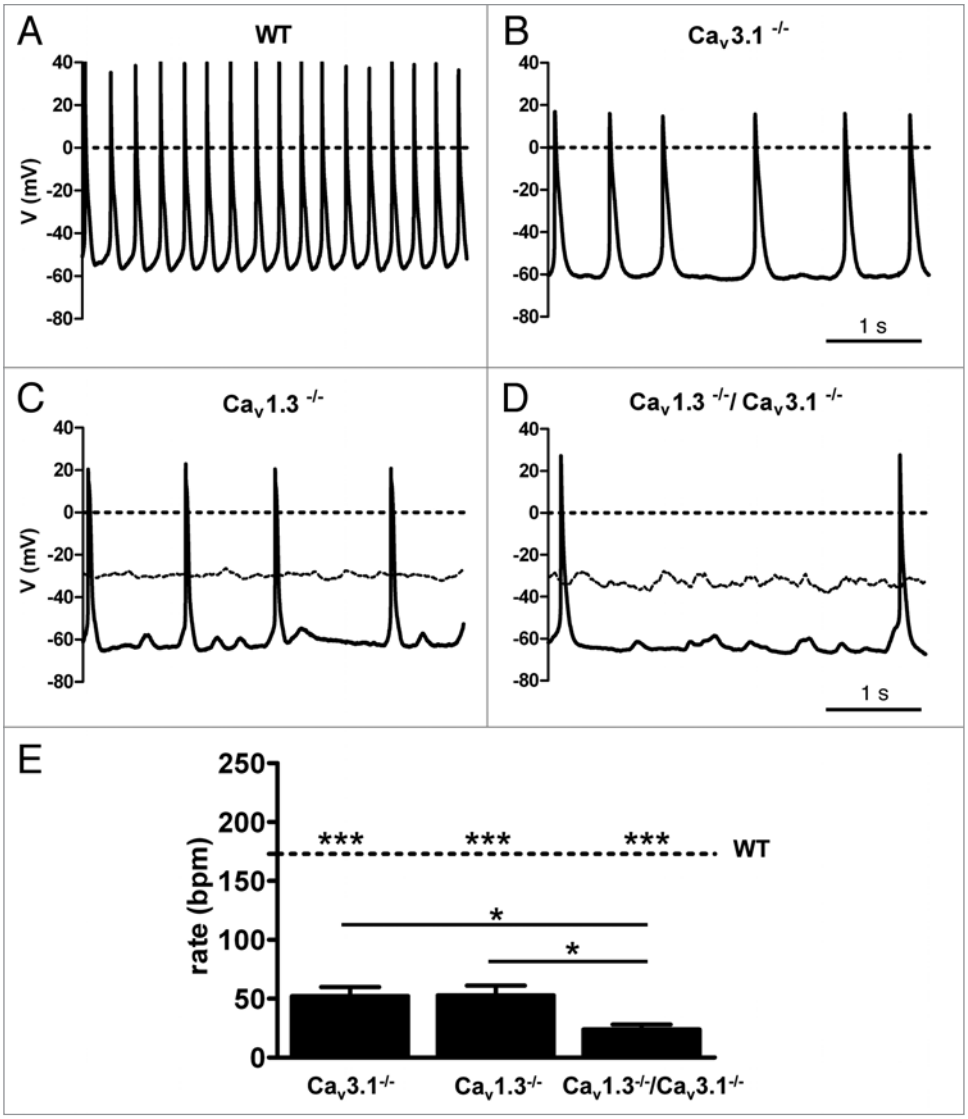

Figure 4. Pacemaker activity of AVNCs from WT (A), $Ca_v3.1^{-/-}$ (B), $Ca_v1.3^{-/-}$ (C) and $Ca_v1.3^{-/-}/Ca_v3.1^{-/-}$ (D) mice. Cells from $Ca_v1.3^{-/-}$ and $Ca_v1.3^{-/-}/Ca_v3.1^{-/-}$ mice were injected with tonic negative hyperpolarizing current (see text). Dashed lines indicate the spontaneous membrane potential before starting current injection. Inactivation of $Ca_v3.1$ channels significantly slowed AVNCs pacemaking and produced erratic beating rate. Only sporadic actions potentials could be observed in negative-current injected $Ca_v1.3^{-/-}$ and $Ca_v1.3^{-/-}/Ca_v3.1^{-/-}$ AVNCs. (E) Histogram showing comparison between averaged beating rates of $Ca_v1.3$, $Ca_v3.1^{-/-}$ and $Ca_v1.3^{-/-}/Ca_v3.1^{-/-}$ AVNCs. The dotted line indicates the averaged beating rate of WT AVNCs. Stars indicate statistical significance (one-way ANOVA).

lines of evidence support this. First, under normal conditions (no injection of constant hyperpolarizing current), $Ca_v1.3^{-/-}$ AVNCs did not display spontaneous automaticity and, even in the presence of saturating doses of ISO, pacemaking could not be restored. Second, even in negative current-injected $Ca_v1.3^{-/-}$ AVNCs, we recorded low pacing frequencies and irregular AP generation. Interestingly, the diastolic depolarization was absent and the membrane voltage of $Ca_v1.3^{-/-}$ AVNCs during the diastolic phase showed mainly subthreshold membrane potential oscillations. This observation indicates that $Ca_v1.3$ channels are a critical determinant in the generation of the diastolic depolarization in AVNCs. Third, while initiated firing of negative current-injected $Ca_v1.3^{-/-}$ AVNCs did accelerate under ISO, maximal rates were still much lower than those of WT counterparts (**Fig. 4**). We did not directly investigate the reason for the positive resting membrane potential in $Ca_v1.3^{-/-}$ AVNCs. A likely hypothesis is that the loss of crosstalk between $Ca_v1.3$ channels and SK2 K^+ channels[13] in $Ca_v1.3^{-/-}$ AVNCs induces depolarization of the membrane resting potential. The observation that $Ca_v1.3^{-/-}$ AVNCs lacked spontaneous automaticity when isolated in vitro may not imply unexcitability in vivo. Indeed, we showed that $Ca_v1.3^{-/-}$ AVNCs are still able to fire I_{Na}-dependent action potentials upon membrane hyperpolarization (**Fig. 2**). It is possible that in the intact AVN, $Ca_v1.3^{-/-}$ myocytes are also sufficiently hyperpolarized to enable I_{Na}-dependent action potentials to be triggered by SAN impulse. In vivo, the both the SAN and AVN are subject of the hyperpolarizing load imposed by the electrical coupling with the right atrium. This phenomenon explains,

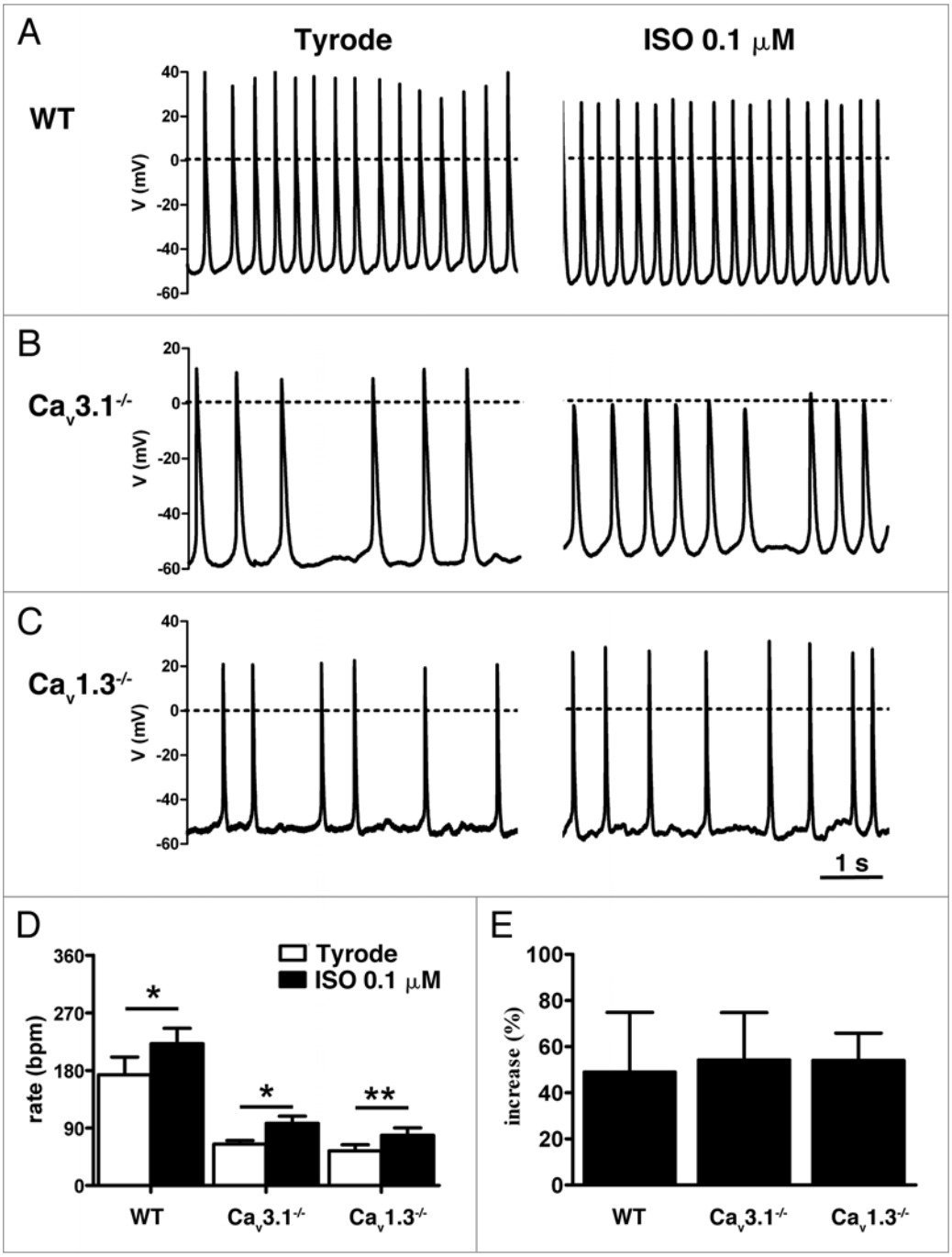

Figure 5. β-adrenergic modulation of firing in AVNCs from WT (A), $Ca_v3.1^{-/-}$ (B) and $Ca_v1.3^{-/-}$ mice (C). AVNCs were challenged with a saturating dose of ISO (0.1 μM). ISO cannot compensate for cellular arrhythmia in $Ca_v3.1^{-/-}$ cells (B). (D) Histogram showing maximum firing rates of WT, $Ca_v3.1^{-/-}$ and $Ca_v1.3^{-/-}$ AVNCs under ISO. Stars indicate statistical significance (two-way ANOVA). (E) The relative sensitivity of pacemaker activity to ISO was similar in WT, spontaneously pacemaking $Ca_v3.1^{-/-}$ and negative current-injected $Ca_v1.3^{-/-}$ AVNCs. The diagram shows that the relative increase in firing rate is constant in these genotypes. Significance was assessed with the one-way ANOVA test.

for instance, why I_{Kr} blockers completely stop pacemaker activity of isolated SANCs, but not in rabbit SAN-atrial preparations[19] or in intact mouse hearts.[20] Hyperpolarizing load by the right atrium could also explain why in another $Ca_v1.3^{-/-}$ mouse line, Zhang and co-workers[21] measured residual AVN automaticity in intact atrial preparations containing the AVN. Finally, it is worth noting that the AVN is a highly heterogeneous structure in which different cell types, possibly serving different conduction pathways can co-exist. Cell-cell interactions between automatic and non-automatic AVNCs, together with the alternative use of different conduction pathways could also explain why $Ca_v1.3^{-/-}$ mice do not show chronic complete AV block. However, the importance of $Ca_v1.3$ channels in AV conduction is stressed by the presence of dissociated rhythms in SNDD patients.[7]

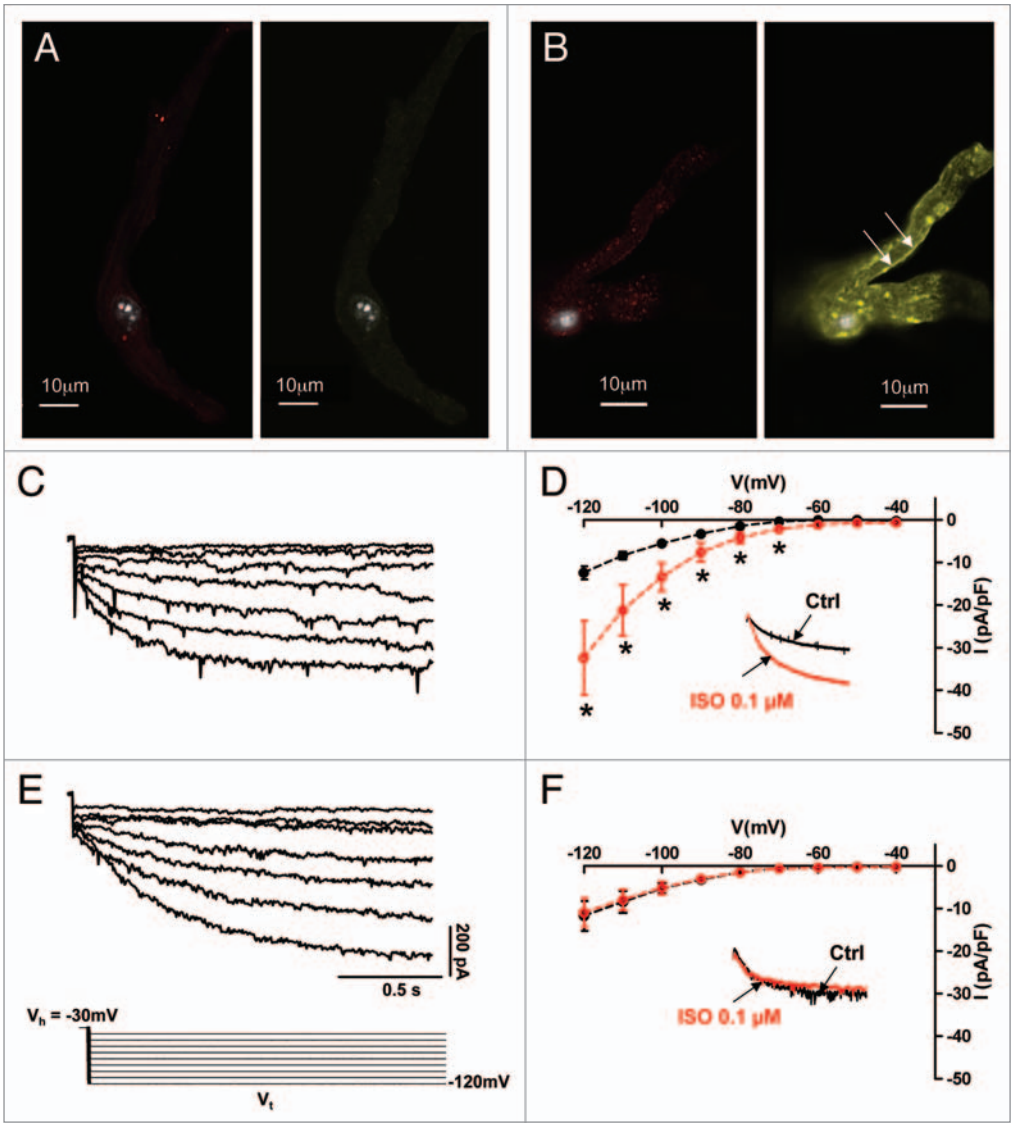

Figure 6. Conditional heart-specific expression of hHCN4-573X transgene suppressed regulation of HCN-mediated I_f by ISO in AVNCs. Staining of cells with an anti-HA antibody evidences expression of hHCN4-573X in AVNCs of mutant mice (A and B). AVNCs from control mice (A) showed only anti HCN4 staining (left part, red) because of expression of endogenous HCN4 channels. No significant anti-HA immunoractivity was observed in control AVNCs. One of three representative experiments is illustrated. In contrast, AVNCs from mutant mice (B), had stronger anti-HCN4 immunoreactivity (red) than control AVNCs and membrane bound anti-HA staining (arrows). I_f sample traces (C) and isochronal I–V curves (D) in control solution (black symbols) and 0.1 μM ISO (red symbols) showed normal responsiveness of I_f to ISO in AVNCs from control mice. In contrast, mutant AVNCs showed that hHCN4-573X expression slowed I_f activation kinetics (E) and abolished I_f sensitivity to ISO (F). Stars indicate statistical significance (Student's t-test). The voltage-clamp protocol of (C and E) is shown. Insets in parts (D and F) show the effect of 0.1 μM ISO in sample traces recorded at -100 mV.

Co-expression of both $Ca_v1.3$- and $Ca_v1.2$-mediated $I_{Ca,L}$ has been reported in SANCs of two different $Ca_v1.3^{-/-}$ mouse strains.[10,16] We estimated in a previous study that in mouse SANCs $Ca_v1.3$-mediated $I_{Ca,L}$ accounts for about 70% of the total $I_{Ca,L}$.[16] In mouse AVNCs functional expression of $Ca_v1.3$ channels appears to be predominant, since inactivation of $Ca_v1.3$ channels reduced AVNC $I_{Ca,L}$ by about 90%. The very low residual $I_{Ca,L}$ density in $Ca_v1.3^{-/-}$ AVNCs prevented us to directly measure the half activation and kinetics of $Ca_v1.2$-mediated $I_{Ca,L}$.

The prominent role of I_{Na} in AVNC AP firing (see Fig. 3 of the accompanying paper in ref. 17) suggests that this current is a major contributor of APs in $Ca_v1.3^{-/-}$ cells. It is possible that, especially under ISO stimulation, residual $Ca_v1.2$-mediated $I_{Ca,L}$ can contribute to the AP upstroke. We thus propose that the absence of basal pacemaker activity in $Ca_v1.3^{-/-}$ cells is not due to incapability of generating the AP upstroke, but rather to insufficient $Ca_v1.3$-mediated $I_{Ca,L}$ in the diastolic depolarization range. In this respect, we performed pilot numerical simulations that suggest that the difference in density and activation of $Ca_v1.3$-mediated $I_{Ca,L}$ could contribute to the dependence of AVNCs pacemaking from I_{Na} (**Sup. Fig. S2**). Thus, the phenotype of $Ca_v1.3^{-/-}$ AVNCs differed from that of $Ca_v3.1^{-/-}$ SANCs, which paced at lower rates than WT cells but retained residual automaticity.[16] Zhang and co-workers[21] recorded $I_{Ca,L}$ using a distinct

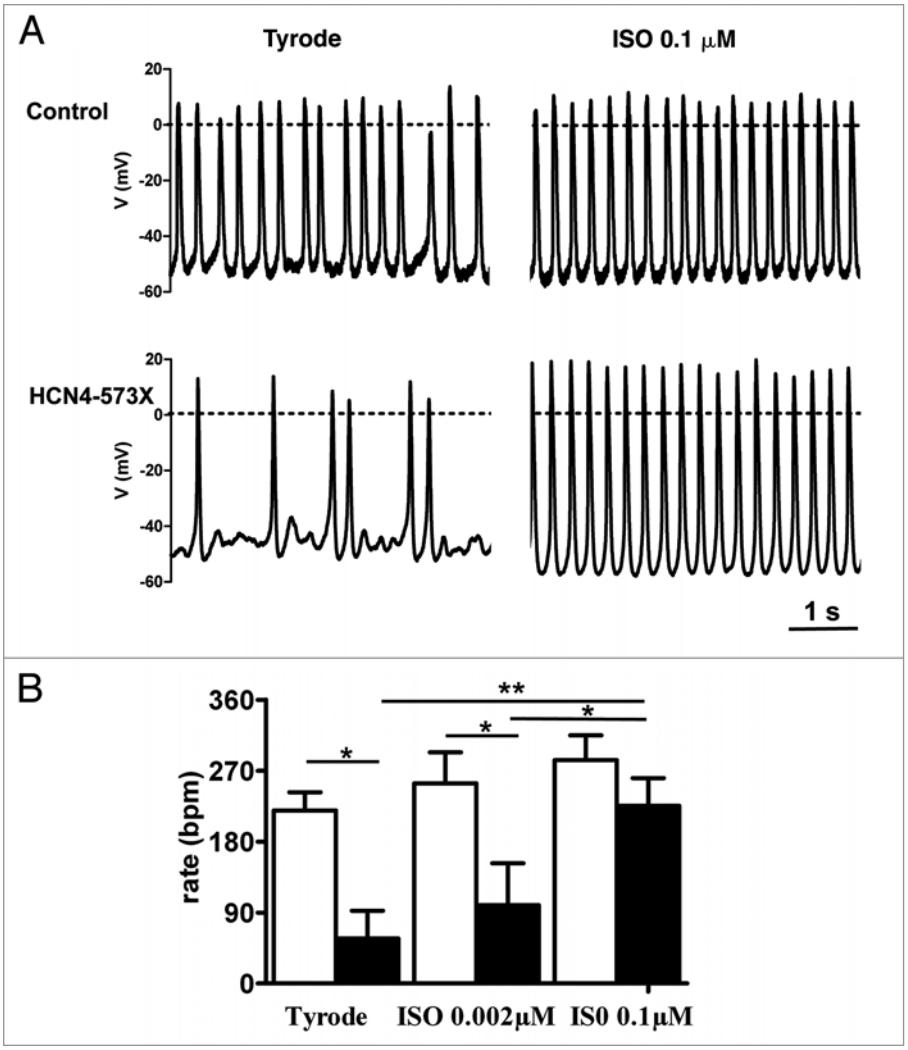

Figure 7. (A) Pacemaker activity in AVNCs from transgenic control mice (upper line) and mice expressing hHCN4-573X (bottom line). Control cells showed regular pacemaker activity in both control conditions and under stimulation by saturating ISO concentration, while AVNCs expressing hHCN4-573X channels showed slow and irregular pacemaking at rest with sporadic AP discharge. ISO induced regular pacemaking in mutant AVNCs. (B) Histogram showing averaged beating rates of control and mutant HCN4-573X AVNCs in basal conditions (Tyrode), after application of a submaximal (0.002 μM) and saturating (0.1 μM) dose of ISO. Pacemaking of HCN4-573X cells reached that of control cells at saturating concentrations of ISO (0.1 μM). Stars indicate statistical significance (two-way ANOVA).

$Ca_v1.3^{-/-}$ mouse line and did not find differences in current densities between WT and $Ca_v1.3^{-/-}$ SANCs[10] or AVNCs.[21] These authors attributed the lack of change in $I_{Ca,L}$ densities to compensation by $Ca_v1.2$ channels in $Ca_v1.3^{-/-}$ SANCs[10] and AVNCs.[21] Discrepancies between results by Zhang et al. and ours may be due to the different genetic background of our $Ca_v1.3^{-/-}$ line and/or to the different strategy of genetic targeting of the $Ca_v1.3$ gene.

Role of $Ca_v3.1$ channels in AVNCs pacemaking. The loss of $Ca_v3.1$-mediated $I_{Ca,T}$ had strong consequences on AVNC automaticity. Indeed, pacemaking of $Ca_v3.1^{-/-}$ AVNCs was intermittent and slower than that of WT cells. Stimulation by ISO augmented the pacemaking rate of $Ca_v3.1^{-/-}$ AVNCs, but maximal rates measured under these conditions were lower than that of WT cells. Interestingly, negative current-injected AVNCs from $Ca_v1.3^{-/-}/Ca_v3.1^{-/-}$ mice had slower firing rate than negative current-injected $Ca_v1.3^{-/-}$ cells, indicating that $Ca_v3.1$ channels can contribute also to initiated automaticity of negative current-injected $Ca_v1.3^{-/-}$ AVNCs. The phenotype of $Ca_v3.1^{-/-}$ cells demonstrates that $Ca_v3.1$-mediated $I_{Ca,T}$ plays an important role in automaticity of AVNCs, by contributing to basal and maximal (under ISO) pacemaking rates. The mechanism of cellular dysrhythmia in $Ca_v3.1^{-/-}$ AVNCs is not known. ISO was only partially effective in increasing pacemaking rates and did not normalize cellular arrhythmia.

HCN channels in AVNCs pacemaking. HCN channels are considered to play a key role in both the generation and regulation of cardiac pacemaker activity.[14] Because current-injected $Ca_v1.3^{-/-}/Ca_v3.1^{-/-}$ AVNCs are still responsive to ISO, we evaluated the role of cAMP-dependent regulation of HCN channels in AVNCs automaticity. To avoid embryonic lethality due to constitutive loss of HCN4 protein[22] we employed our new mouse model, which conditionally expresses the HCN4-573X transgene abolishing cAMP sensitivity of HCN channels in a dominant negative way.[15] A striking finding is that the loss of cAMP dependent regulation of HCN channels induced slow and irregular pacemaker activity in mouse AVNCs. However, ISO application raised regular pacemaking of mutant AVNCs and maximal rates of control and mutant AVNCs were comparable. Our results thus indicate that HCN channels are important for basal excitability of mouse AVNCs, but are not required for acceleration of pacemaker activity under β-adrenergic receptor stimulation by ISO and for reaching maximal pacemaking rates. This result is in agreement with a recent study that showed no effect of the f-channel blocker ZD-7288 on ISO-induced acceleration of pacemaker activity in isolated canine AVN preparations.[23] The preserved β-adrenergic regulation of AVNCs pacemaking observed in $Ca_v1.3^{-/-}$, $Ca_v3.1^{-/-}$ and HCN4-573X expressing AVNCs is intriguing. One possibility is that other mechanisms such as RYR-dependent Ca^{2+} release[24] or CaMKII,[25] mechanisms that are operant in SANCs can mediate regulation of AVNCs pacemaking in mutant AVNCs. Also, we cannot exclude that residual $Ca_v1.2$-mediated $I_{Ca,L}$ can compensate, in part, for the lack of $Ca_v1.3$ channels in $Ca_v1.3^{-/-}$ AVNCs.

Conclusions and implications. Our work indicates that besides well known pacemaker mechanism such as HCN-channels and

RYR-dependent Ca^{2+} release,[23,24] $Ca_v1.3$ and $Ca_v3.1$ channels are important for AVNCs automaticity. A recent pharmacological study on intact AVNs by Lu et al. suggested that the relative importance of ion channels and RyR-dependent Ca^{2+} release in pacemaking can vary between the SAN and AVN.[26] In this respect, our results show important differences in the impact of $Ca_v1.3$, $Ca_v3.1$ and HCN channels between AVNCs and SANCs. A preliminary numerical model of AVNCs automaticity based on $Ca_v1.3$, $Ca_v3.1$ and HCN channels is provided in the supplemental online section. Interestingly, it has been reported that AVNC precursors show a distinct genetic program than that of SAN.[27] It is thus tempting to speculate that such a differential genetic program is reflected into differences in the underlying pacemaker mechanism.

Materials and Methods

The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US national Institute of Health (NIH Publication No. 85–23, revised 1996) and European directives (86/609/CEE).

Generation of mouse lines. Mice lacking $Ca_v1.3$ channels ($Ca_v1.3^{-/-}$) of C57B/6J genetic background were obtained as described previously in reference 16. Mice lacking $Ca_v3.1$ channels ($Ca_v3.1^{-/-}$) were derived from $Ca_v3.1^{-/-}$ mice on 129Sv genetic background[11] that were backcrossed on C57B/6J mice for more than ten generations. $Ca_v1.3^{-/-}/Ca_v3.1^{-/-}$ mice were obtained by crossing C57B/6J $Ca_v1.3^{-/-}$ with $Ca_v3.1^{-/-}$ mice. Mice expressing hHCN4-573X channels in a conditional and heart specific way were obtained as described previously in reference 15. Briefly, mice carrying an alpha myosin heavy chain (αMHC) and Tet-Off system controlled promoter transgene were used as controls (αMHC-tTA mice). Double transgenic mutant mice were obtained by crossing αMHC-tTA mice with transgenic mice carrying an engineered human HCN4 cDNA (hHCN4) that lacked the cAMP-binding domain and contained an hemagglutinin (HA) sequence tag at the N-terminus (hHCN4-573X). Breeding mice in doxycycline-free drinking water induced expression of hHCN4-573X channels.

Telemetric ECG recordings. ECGs recording were performed in control and mutant mice as described previously in reference 11. An expanded description of ECG recording and analysis is available in the online data supplement (Sup. online methods).

Isolation of mouse AVNCs and patch-clamp recordings. AVN and SAN tissues were obtained from female mice aged of 2–4 months as described in the accompanying paper in reference 17. All electrophysiological experiments were carried out at 36°C. The whole-cell variation of the patch-clamp technique was used to measure Ca^{2+} currents. All other experiments have been performed using the perforated patch technique with β-escin (50 μM).

Statistical analysis. Results are presented as means ± standard error of the mean (SEM, number of cells). For calculating the level of significance, the Student's t-test, the one- or two-way ANOVA tests followed by Tukey's post-hoc test or the non-parametric Kruskal-Wallis test have been employed. When testing statistical differences results were considered significant with $p < 0.05$. In all figures $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$, respectively.

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Note

Supplementary materials can be found at:
www.landesbioscience.com/journals/channels/article/15266

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