Pacemaker activity and ionic currents in mouse atrioventricular node cells

Laurine Marger,1,3 Pietro Mesirca,1,3 Jacqueline Alig,4 Angelo Torrente,1,3 Stefan Dubel,1,3 Birgit Engeland,4 Sandra Kanani,1,3 Pierre Fontanaud,1b-3 Jörg Striessnig,3 Hee-Sup Shin,6 Dirk Isbrandt,4 Heimo Ehmk,7 Joël Nargeot1,3 and Matteo E. Mangoni1,3,8

1CNRS, UMR-5203; Institut de Génomique Fonctionnelle; Département de Physiologie; 2Département d’Endocrinologie; Montpellier, France; 3Département d’Endocrinologie; Montpellier, France; 4Institute of Pharmacology and Toxicology; University Medical Center Hamburg-Eppendorf; Hamburg, Germany; 5Department of Pharmacology and Toxicology; Institute of Pharmacy; Center for Molecular Biosciences; University of Innsbruck; Innsbruck, Austria; 6Center for Neuro Science; Korea Institute of Science and Technology; Cheonggye, Seoul, Republic of Korea

Key words: atrioventricular node, sino-atrial node, pacemaker activity, ion channels, electrophysiology, conduction, heart rate, Ca2+ channels, Na+ channels, f-channels, K+ channels

Abbreviations: AVN, atrioventricular node; SAN, sino-atrial node; TTX, tetrodotoxin; INa, voltage-dependent Na+ current; ICa,L, L-type Ca2+ current; ICa,T, T-type Ca2+ current; If, hyperpolarization-activated f-current; IKr, delayed rectifier K+ current; IKo, transient outward K+ current

It is well established that pacemaker activity of the sino-atrial node (SAN) initiates the heartbeat. However, the atrioventricular node (AVN) can generate viable pacemaker activity in case of SAN failure, but we have limited knowledge of the ionic bases of AVN automaticity. We characterized pacemaker activity and ionic currents in automatic myocytes of the mouse AVN. Pacemaking of AVN cells (AVNCs) was lower than that of SAN pacemaker cells (SANCs), both in control conditions and upon perfusion of isoproterenol (ISO). Block of If by tetrodotoxin (TTX) or of ICa,L by isradipine abolish AVNCs pacemaker activity. TTX-resistant (I(Ka)) and TTX-sensitive (I(Ks)) Na+ currents were recorded in mouse AVNCs, as well as T- (ICa,T) and L-type (ICa,L) Ca2+ currents. ICa,L density was lower in SANCs (51%). The density of the hyperpolarization-activated current (I(f)) and that of the fast component of the delayed rectifier current (I(Kr)) were, respectively, lower (52%) and higher (53%) in AVNCs than in SANCs. Pharmacological inhibition of If by 3 μM ZD-7282 reduced pacemaker activity by 16%, suggesting a relevant role for If in AVNCs automaticity. Some AVNCs expressed also moderate densities of the transient outward K+ current (I(Ko)). In contrast, no detectable slow component of the delayed rectifier current (I(Kr)) could be recorded in AVNCs. The lower densities of If and ICa,L as well as higher expression of ICa,L in AVNCs than in SANCs may contribute to the intrinsically slower AVNCs pacemaking than that of SANCs.

Introduction

The sino-atrial node (SAN) controls the heart rhythm and rate under physiological conditions.1 The AVN is also endowed with automaticity. It is generally accepted that the AVN drives the heartbeat as a “secondary” supraventricular pacemaker in case of SAN failure.2,3 Due to its pivotal role in cardiac conduction and as a secondary pacemaker, the structure and the function of the AVN have been extensively studied (recently reviewed in ref. 3). However, little is known about mechanisms underlying AVN automaticity. Some ion channels known to be involved in SAN pacemaker activity have been found in the AVN,4,5 but their importance in the generation of automaticity in AVNC has not been investigated in depth at the cellular level. Recently, the phenotypes of genetically-modified mouse strains in which genes coding for ion channels involved in cardiac automaticity have been described (reviewed in ref. 6). Genetically-modified mice constitute a promising approach to understand the bases of AVN automaticity, but the electrophysiological properties of spontaneously active mouse AVN cells (AVNCs) have not been investigated directly and we have limited knowledge on the functional role of ionic currents in mouse AVNCs. The aim of the present study was to isolate spontaneously active mouse AVNCs to characterize the properties of AVNCs pacemaker activity, as well as to record the major ionic currents potentially involved in AVNCs automaticity.

Mouse AVNCs displayed an intrinsically slower pacemaker activity than that of SANCs. In comparison to adult rabbit2 and mouse5 SANCs, automaticity of mouse AVNCs depended on If. A higher density than in SANCs of the fast component of the delayed rectifier current (I(Kr)) also characterized mouse AVNCs. Densities of the L-type Ca2+ currents (ICa,L) were lower in AVNCs than in SANCs. Mouse AVNCs expressed lower densities of the hyperpolarization-activated current (I(f)) than SANCs. If showed
tissue preparations with an anti-rat HCN4 antibody (Fig. 1A and B). HCN4 positive (presumably pacemaker) AVNCs were present in our AVNC preparations (Fig. 1A and B). In particular, anti-HCN4 immunoreactivity was identified in the SAN as stretches of HCN4-positive cells extending into the AVN region below the inferior vena cava (Fig. 1A). This is consistent with what was reported by Dobrzynski et al.\textsuperscript{10} who delineated expression of HCN4 protein in rabbit AVN. Beside the posterior nodal extension of the conduction system identified by Dobrzynski et al.\textsuperscript{10} our preparations included also the enclosed node, and a AVN sub-region defined by Boyett et al.\textsuperscript{11} that was composed by “loosely-packed” atrial cells. Our preparations did not include cells of the mouse His bundle, because the caudal cutting edge of our tissue samples was located on the tricuspid valve. We also checked in pilot experiments using Cx40\textsuperscript{EGFP/+} mice\textsuperscript{12} that the His bundle was left intact after cutting out the AVN region (see Fig. 2B in the paper by Miquerol et al.\textsuperscript{12}). We have thus used all these landmarks to perform our dissections so to include the full mouse AVN region.

Our next objective was to identify the functional signature of “pacemaker” AVNCs in mice. Three cellular morphologies

![Figure 1](https://example.com/image1.png)

**Figure 1.** (A) Whole SAN-AVN preparation stained with an anti-rat HCN4 antibody effectively showed the organization of HCN4 positive SANCs and AVNCs. HCN4 positive cells are visible as stretches of cell groups along the crista terminalis and then extending in the posterior part of the lumen of the inferior vena cava. The coronary sinus was cut open for clarity. Distribution of HCN4 immunoreactivity corresponds to the extension of the HCN4-positive conduction system recently described by Dobrzynski et al. in the rabbit AVN.\textsuperscript{10} (B) HCN4-positive spindle AVNCs can be seen in a close-up view of the AVN region shown in (A). Abbreviations: right atrium (RA), left atrium (LA), aorta (Ao), crista terminalis (CT); superior vena cava (SVC); inferior vena cava (IVC); coronary sinus (CS); Fossa ovalis (FO); atrioventricular node (AVN); sino-atrial node (SAN). (C) Morphology of myocytes isolated from the mouse AVN. Rod-shaped cells (middle part) and atrial cells (right part) displayed no pacemaker activity. Spindle shaped cells (left part) were beating and were named AVNCs in this study. Note the morphological similarity between the morphology of isolated AVNCs and HCN4 positive cells in (B).
Spindle- and rod-shaped cells have been reported in the rabbit AVN by Munk et al. and by Hancox and Levi, respectively. In our mouse AVN preparations, rod-shaped and atrial-like cells did not appear to be spontaneously active. However, we cannot exclude the possibility that rod-shaped cells can become automatic under particular physiological conditions or in situ in the intact AVN.

Only spontaneously active spindle-shaped cells were used for this study (Fig. 1C). The mean input capacitance of n = 147 spindle shaped cells was 20 ± 1 pA/pF, a value similar to that reported for primary SANCs (20 pF).13 Spindle-shaped cells from AVN of the mouse (this study), the rabbit7 and guinea pig14 have comparable cellular input capacitance (22 pF in mouse, 29 pF in rabbit and 25 pF in guinea pig). The presence of rod-shaped and atrial-like cells in preparations is consistent with the cellular heterogeneity described in the rabbit AVN.

Pacemaking of mouse AVNCs is slower that of SANCs and depends on both $I_{Na}$ and $I_{Ca,L}$. Comparison between pacemaker activity of isolated AVNCs and SANCs showed that the beating rate of mouse AVNCs was significantly lower than that of SANCs (Table 1). The maximum diastolic potential and the action potential threshold were significantly more negative in AVNCs than in SANCs (Table 1) indicating that the diastolic depolarization occurs at a more negative voltage range in AVNCs than in SANCs. To test if ISO application could compensate for the slower basal rate of AVNCs, we applied ISO at 0.1 μM, a concentration that largely saturated the positive chronotropic response to this agonist of mouse SANCs pacemaking (data not shown). The cellular firing rate measured after application of ISO was still higher in SANCs (C) than in AVNCs (D). In ISO, the beating rate of SANCs was 348 ± 7 bpm, while that of AVNCs measured in the same conditions was 222 ± 24 bpm (p < 0.05).

Because genetic mutations affecting the cardiac $I_{Na}$ channel isoform Na1.5 and the L-type Ca1.3 channel isoform cause AV conduction dysfunction,5,15 we investigated the impact of inhibition of $I_{Na}$ and $I_{Ca,L}$ on AVNCs pacemaking. Application of 20-μM tetrodotoxin (TTX) blocked action potential discharge (Fig. 3A). The membrane potential of AVNCs exposed to 20 μM TTX was stable at -59 ± 2 mV (n = 8). Inhibition of $I_{Ca,L}$ by 0.3 μM of the L-type channel blocker isradipine stopped pacemaker activity of AVNCs and the cell membrane potential depolarized to -35 ± 3 mV (n = 6). Only low amplitude oscillations of the membrane potential could be observed in isradipine treated AVNCs (Fig. 3B). These results indicated that pacemaking of mouse AVNCs required both $I_{Na}$ and $I_{Ca,L}$ for action potential discharge.

---

**Table 1. Pacemaker activity and action potential parameters of mouse AVNCs and SANCs**

|            | SANCs | AVNCs |
|------------|-------|-------|
| Rate (bpm) | 260 ± 21 | 173 ± 27 |
| MDP (mV)   | -53 ± 1  | -57 ± 1  |
| Eth (mV)   | -35 ± 1  | -41 ± 2  |
| SDD (mV/ms)| 0.09 ± 0.1 | 0.05 ± 0.01 |
| APA (mV)   | 88 ± 7  | 91 ± 7  |
| dV/dt (mV/ms)| 14 ± 5  | 13 ± 3  |
| APD (ms)   | 134 ± 12 | 152 ± 18 |

AVNCs displayed slower beating rate (bpm), a more negative maximum diastolic potential (MDP) and action potential threshold (Eth). The slope of the diastolic depolarization (SDD), was lower in AVNCs. No significant differences were observed in the action potential amplitude (APA), upstroke velocity (dV/dt) and action potential duration (APD).

---

**Figure 2.** Automaticity of WT AVNCs is slower than that of SANCs. (A) Examples of spontaneous action potentials in AVNCs and SANCs; consecutive action potentials are superimposed for clarity. The dotted line sets the zero-voltage level. (B) Histograms of pacing rate of WT mouse SANCs (filled boxes) and AVNCs (empty boxes). Perfusion of a maximal dose of ISO (0.1 μM) increased pacemaking of SANCs (C) and AVNCs (D), but AVNCs pacemaking was still slower than that of SANCs under the same condition.
Voltage-dependent Na⁺ currents in mouse AVNCs. Both TTX-sensitive ($I_{Na,s}$) and TTX-resistant ($I_{Na,r}$) Na⁺ currents have been recorded in mouse SANCs.⁶⁻⁷ To test functional expression of $I_{Na}$, we applied 0.1 μM TTX to inhibit this current.⁶ At this concentration, total peak $I_{Na}$ was reduced by about 20% (Fig. 4). The net peak density of $I_{Na}$ blocked at 0.1 μM TTX at a test potential of -25 mV was (56 ± 12 pA/pF, n = 8), while that of residual $I_{Na}$ was 287 ± 57 pA/pF, n = 8. Residual $I_{Na}$ after application of 0.1 μM TTX was not completely blocked at 20 μM TTX (Fig. 3B), demonstrating that this current is attributable to $I_{Na,s}$, $I_{Na,t}$, and $I_{Na,r}$, displayed distinct $V_{1/2}$. Activation of $I_{Na,s}$ was 11 mV negative to that of $I_{Na,t}$. This is consistent with a previous study in mouse SANCs.⁷ The $V_{1/2}$ of $I_{Na,t}$ was significantly more positive than that of $I_{Na,r}$ (-32 ± 1 mV for $I_{Na,t}$ and -43 ± 3 mV for $I_{Na,r}$, p < 0.05, Fig. 4C).

$I_{Ca,L}$ and $I_{Ca,T}$ in mouse AVNCs. Beside $I_{f}$, another typical hallmark of pacemaker SANCs is co-expression of "low-voltage activated" T- and L-type Ca²⁺ channel isoforms.⁸⁻⁹ We thus measured $I_{Ca,L}$ and $I_{Ca,T}$ in AVNCs (Fig. 5). The peak current density of $I_{Ca,T}$ measured at the test potential of -30 mV was 7.9 ± 0.7 pA/pF, a value which is significantly higher than that of the peak $I_{Ca,L}$ (1.7 ± 0.1 pA/pF, n = 4, $V_{1/2}$ -5 mV). The $V_{1/2}$ of $I_{Ca,T}$ was -45 ± 1 mV, while that of $I_{Ca,L}$ was -22 ± 2 mV. Current densities did not significantly differ between WT C57B/6J mice (this study) and that recorded in AVNCs of 129Sv mice.¹⁰

Properties of $I_{f}$ in mouse AVNCs. $I_{f}$ was present in all spontaneously-beating AVNCs recorded. The density of $I_{f}$ AVNC pacemaker activity by about 16% (Fig. 7B). At this concentration ZD-7228 slowed the automaticity of WT AVNCs is dependent on both $I_{Na}$ and $I_{f}$. (A) Representative recordings of consecutive action potentials of WT mouse AVNCs in control conditions and after inhibition of $I_{Na}$ by 20 μM TTX (B).
Discussion

The AVN structure and function have been extensively studied (reviewed in ref. 3) however, little is known about the mechanisms underlying AVN automaticity. In this study we present, for the first time, an overview of the electrophysiological properties of spontaneously active mouse AVNCs. We expect that our work will improve the accessibility of this preparation for future studies on AVN pacemaker activity and conduction using genetically engineered mice. Furthermore, our results provide a functional signature of automatic mouse AVNCs and can be useful for studies aiming to characterize AVNCs precursors from the developing mouse heart or from differentiating stem cells.

Pacemaking of mouse AVNCs. The AVN is a highly heterogeneous structure containing different cell types that probably serve both SAN impulse conduction and secondary pacemaker activity. Since little is known about the specific cellular mechanisms underlying AVNCs automaticity, we focused on AVNCs that displayed pacemaker activity once isolated from the intact AVN. In addition to widely accepted anatomical landmarks established in rabbit hearts (see the Materials and Methods section), we used also anti-HCN4 staining of intact AVN to identify regions of the AV junction containing automatic cells. This approach maximizes the probability of including all automatic AVNCs in our preparations, even if the fine structure of mouse AVN has not been fully explored. Even if we did not perform a pilot structure-to-function study of mouse AVNCs, we found consistency between the anatomical pattern of anti-HCN4 immunoreactivity in our preparations and previous studies on rabbit conduction system.25 Dobrzynski et al.10 have reported that the site of origin of automaticity in the rabbit AVN is predominantly located in the posterior nodal extension, even if automaticity can also originate in the enclosed node region. In our preparations, we included the mouse posterior nodal extension, which is HCN4 positive and is thus homologous to the rabbit AVN rhythmogenic centre (Fig. 1A). Also, the gross morphology of HCN4 positive cells in situ is similar to the spindle-like shaped AVNCs used for recordings (Fig. 1B and C). In conclusion, we are confident that spontaneously beating AVNCs described in this study come from rhythmogenic centers of AVN and underlie its automaticity in vivo.

- The intrinsic pacemaker activity of mouse AVNCs was significantly slower than that of SANCs (Fig. 2). This difference was maintained after perfusion with ISO. This observation indicates that the slower rate of pacing of mouse AVNCs cannot be explained with a lower basal cAMP concentration, but is due to intrinsic properties of the AVNCs pacemaker mechanism.

\[ I_{Na} \text{ in mouse AVNCs.} \]
Automaticity of mouse AVNCs was highly sensitive to \( I_{Na} \) block by TTX. \( I_{Na} \) thus appears as the predominant mechanisms underlying the action potential upstroke phase. In mouse SANCs high doses of TTX do not stop pacemaker activity, but slow the action potential upstroke velocity as well as the diastolic depolarization phase.8 This suggests that the functional role of \( I_{Na} \) in mouse AVNCs can be quantitatively different from that in SANCs. Similarly to SANCs, AVNCs expressed both \( I_{Na,r} \) and \( I_{Na,s} \). Activation of \( I_{Na,r} \) was 11 mV negative to that of \( I_{Na,s} \). This is consistent with a previous study in mouse SANCs.8 Similarly to SANCs, preliminary data suggest that \( I_{Na,s} \) contributes to the diastolic depolarization of AVNCs, because block of TTX-sensitive channels by 0.1 \( \mu M \) TTX affected the slope of this phase (data not shown). At present, it is difficult to compare the densities of \( I_{Na,r} \) and \( I_{Na,s} \) between AVNCs and SANCs, because in the previous study by Lei et al.8 Na’ currents were recorded at room temperature and under reduced extracellular Na’ concentrations (30 mM). However, the high density of \( I_{Na,s} \) that we recorded in AVNCs and the prominent role of \( I_{Na,s} \) in AVNCs action potential discharge are consistent with the severe dysfunction in AVN conduction observed in haplo-insufficient
However, in spite of the strong expression of $I_{\text{Na}}$, $I_{\text{Ca,L}}$ is functionally important in AVNCs, because isradipine completely stopped action potential discharge. The absolute density of $I_{\text{Ca,T}}$ in mouse AVNCs was significantly higher than that of $I_{\text{Ca,L}}$ (Fig. 4). This contrasts to what previously observed in rabbit SANCs, where $I_{\text{Ca,L}}$ density is higher than that of $I_{\text{Ca,T}}$.18,28 High expression of $I_{\text{Ca,T}}$ in AVNCs, can be an adaptative mechanism to sustain both high basal pacemaking and fast AV conduction in mouse AVN. Use of genetically-modified mice will be useful to understand the functional role of $I_{\text{Ca,L}}$ and $I_{\text{Ca,T}}$ in AVNCs automaticity.

Expression of $I_{\text{Na}}$ in AVNCs is also consistent with the fast impulse conduction in mouse AVNCs.

$I_{\text{Ca,L}}$ and $I_{\text{Ca,T}}$ in mouse AVNCs. Automaticity of AVNCs also depended from $I_{\text{Ca,L}}$ (Fig. 3B). Perfusion of isradipine blocked action potential discharge and depolarized the membrane potential. AVNCs thus responded to $I_{\text{Ca,L}}$ block in a similar way as central SAN tissue strips from the rabbit heart.26 We propose that depolarization of membrane voltage upon $I_{\text{Ca,L}}$ block can be explained, at least in part, by the loss of coupling between L-type channels and SK2 K+ channels. Such a functional coupling has been reported in mouse AVNCs,24 as well as in atrial myocytes.27 The density of $I_{\text{Ca,L}}$ recorded in AVNCs was significantly lower than that of SANCs (Fig. 4). However, in spite of the strong expression of $I_{\text{Na}}$, $I_{\text{Ca,L}}$ is functionally important in AVNCs, because isradipine completely stopped action potential discharge. The absolute density of $I_{\text{Ca,T}}$ in mouse AVNCs was significantly higher than that of $I_{\text{Ca,L}}$ (Fig. 4). This contrasts to what previously observed in rabbit SANCs, where $I_{\text{Ca,L}}$ density is higher than that of $I_{\text{Ca,T}}$.18,28 High expression of $I_{\text{Ca,T}}$ in AVNCs, can be an adaptative mechanism to sustain both high basal pacemaking and fast AV conduction in mouse AVN. Use of genetically-modified mice will be useful to understand the functional role of $I_{\text{Ca,L}}$ and $I_{\text{Ca,T}}$ in AVNCs automaticity.

$I_{\text{f}}$ in mouse AVNCs. In AVNCs, the density of $I_{\text{f}}$ was significantly lower than that recorded in SANCs (Fig. 5). Activation of $I_{\text{f}}$
was shifted negatively in AVNCs by about 10 mV compared to SANCs and AVNCs. In spite of the weaker density and the more subtle differences exist in the regulation of HCN channels in IKr of AVNCs. The predominance of IKr suggests that this current can be important also for pacemaking function as an outward current that influences the time course of this action potential duration and is present during the diastolic depolarization from 2.56 ± 0.5 to 2.17 ± 0.5, (p < 0.05). The action potential duration was not significantly affected by ZD-7288 (from 286 ± 22 to 242 ± 23 bpm, n = 5, p < 0.05). ZD-7288 reduced the slope of the diastolic depolarization from 2.56 ± 0.5 to 2.17 ± 0.5, (p < 0.05).

Figure 7. Properties of I_f in mouse AVNCs. (A) I_f activation curve in mouse AVNCs, measured according to DiFrancesco and Mangoni. Red circles indicate curve fit simulation. (B) ZD-7228 inhibited I_f by 34.3 ± 7.3% (at -115 mV, n = 5). I_f was activated by hyperpolarizing steps as in Figure 6. (C) ZD-7228 reduced AVNCs beating rate by 16 ± 2% (from 286 ± 22 to 242 ± 23 bpm, n = 5, p < 0.05). ZD-7228 reduced the slope of the diastolic depolarization from 2.56 ± 0.5 to 2.17 ± 0.5, (p < 0.05). The action potential duration was not significantly affected by ZD-7228 (from 301 ± 81 to 336 ± 77 ms at 3 μM ZD-7228, p > 0.05).

Materials and Methods

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

Morphological identification and definition of mouse AVN. The mouse AVN was identified as the region between the inferior vena cava (IVC), the coronary sinus (CS) and the tricuspid valve (Sup. Fig. 1A and B). The anatomical location of tissue samples used for cell isolation is from the mouse atrioventricular junction corresponding to the Koch’s triangle as defined in other mammals (Sup. Fig. 1A and B). This region corresponded to the “AVN region” identified by Rentschler et al. at the inferior part of the interatrial septum and above the mitral annulus. Indeed, in the Rentschler et al. study, hearts from transgenic mice expressing an Engrailed-LacZ reporter construct delineated the mouse cardiac conduction system from the SAN to the distal Purkinje fibers. Finally, we considered as the AVN, the entire region that has been shown to express a pattern of mRNA coding for ion channels similar to that of SAN and containing HCN4-positive cells (see Results section). Indeed, Marionneau et al. have reported that ion channel mRNA expression pattern in the mouse AVN region is more similar to that of SAN, a finding that is consistent with the functional expression of ionic currents identified in the present study (see above).

Staining of AVN tissue. Isolated mouse heart node tissue was placed immediately into 4% paraformaldehyde for 20 minutes at room temperature (RT), followed by soaking in 1x PBS for several hours. The tissue was suspended in 250 ml of 2% Bovine Serum Albumin (Sigma), 500 mg/ml digitonin (Sigma) containing rat HCN4 monoclonal antibody (SHG 1E5, Santa Cruz, 1:100) and sodium azide (0.002%, to prevent bacterial growth) for 66 hrs at RT with very gentle shaking in a LAB-TEK II Chamber Slide. The tissue was rinsed 5 x 500 ml (1x PBS) over a 5-hour period at RT. The procedure was repeated again but this time with donkey anti-rat Alexa 488 secondary (Molecular Probes, 1:500). The tissue was placed in prolong gold (Invitrogen) and a glass coverslip gently placed over the sample. Images were taken with a Leica confocal microscope (Leica TCS LSI) at the Montpellier RIO Imaging facility of the Arnaud de Villeneuve Campus (Montpellier, France). The final images represent a snapshot of a 3D reconstructed tiled image using IMARIS software package (PC, version 7).

Mouse AVNCs were isolated using the same protocol as used for patch clamping. AVNCs were placed into chambers and

www.landesbioscience.com Channels 247
The specificity of Rat HCN4 antibody was confirmed using a mouse monoclonal HCN4 antibody (Neuromab), which had the identical overlapping staining pattern. Furthermore, SANCs expressing HCN4-HA tagged subunits showed overlapping staining using rat HCN4/mouse HA antibodies or mouse HCN4/rat HA antibodies (data not shown).

**Isolation of mouse SANCs and AVNCs.** Beating hearts were removed under general anesthesia, consisting of 0.01 mg/g of Xylazine (Rompun 2%, Bayer AG, Leverkusen Germany), 0.1 mg/g of Ketamine (Imalgène, Merial, Bourgelat France) and 0.2 mg/g of Na-Pentobarbital (CEVA, France). The SAN and the AVN were exposed by using the landmarks shown in Supplemental Figure S1A and B and then excised in pre-warmed (35°C) Tyrode solution containing (mM/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). SAN tissue strips were then transferred into a “low-Ca²⁺-low-Mg²⁺” solution containing (in

![Figure 8.](image-url)
mM/L): NaCl, 140; KCl, 5.4; MgCl₂, 0.5; CaCl₂, 0.2; KH₂PO₄, 1.2; taurine, 50; D-glucose, 5.5; bovine serum albumin (BSA), 1 mg/ml; HEPES-NaOH, 5; (adjusted to pH = 6.9 with NaOH). SAN and AVN tissues were digested by adding collagenase type II or IV (229 U/ml, Worthington Biochemical Corporation, Lakewood, NJ USA), elastase (1.9 U/ml, Worthington Biochemical Corporation, Lakewood, NJ, USA), protease (0.9 U/ml, Sigma, St. Quentin Fallavier, France), BSA 1 mg/ml and 200 μM CaCl₂. Digestion was carried out for a variable time of 20–25 minutes at 37°C. Tissue strips were then washed and transferred into a modified “Kraftbühre” (KB) medium containing (in mM/L): L-glutamic acid, 70; KCl, 20; KOH, 80; (±)D-b-OH-butyric acid, 10; KH₂PO₄, 10; taurine, 10; BSA, 1 mg/ml; and HEPES-KOH, 10; (adjusted to pH = 7.4 with KOH). Single SAN and AVN cells were isolated by manual agitation in KB solution at 37°C for 2–5 minutes.

Cellular automaticity was recovered by re-adapting the cells to a physiological extracellular Ca²⁺ concentration by addition of a solution containing (in mM/L): NaCl, 10, CaCl₂, 1.8 and normal Tyrode solution containing BSA (1 mg/ml). The final storage solution contained (mM/L): NaCl, 100; KCl, 35; CaCl₂, 1.3; MgCl₂, 0.7; L-glutamic acid, 14; (±)D-b-OH-butyric acid, 2; KH₂PO₄, 2; taurine, 2; BSA 1 mg/ml; (pH = 7.4) and gentamicin (50 μg/ml). All chemicals were from Sigma (St. Quentin, Fallavier), except for the (±)D-b-OH-butyric acid that was from Fluka Chemika (Buchs, CH), TTX and ZD 7288 that were from Tocris Bioscience (Ellisville, USA), 293B was a generous gift by Dr. Lang and E-4031 that was a generous gift by Dr. Flavien Charpentier (L’ Institut du Thorax, Nantes).

Electrophysiological recordings. For electrophysiological recordings, aliquots of the cell suspension were harvested in custom made recording chambers (working volume 500 μL) allowing unidirectional flow of solutions and mounted on the stage of an inverted microscope (Olympus, X71), and continuously perfused with normal Tyrode solution. The recording temperature was set to 37°C. The whole-cell variation of the patch-clamp technique was employed to record cellular ionic currents, by employing an Axopatch 200A (Axon Instruments Inc., Foster USA) patch-clamp amplifier, connected to the ground by an agar bridge filled with 150 mM KCl. Cellular automaticity was recorded by the perforated patch technique with eiscn (50 μM). Recording electrodes were fabricated from borosilicate glass, by employing a Flaming-Brown microelectrode puller (Sutter, Novato, CA USA). For recording cell automaticity, as well as If and K⁺ currents, we used an intracellular solution containing (mM/L): K⁺-aspartate, 130; NaCl, 10; ATP-Na⁺ salt, 2; creatine phosphate, 6.6; GTP-Mg²⁺, 0.1; CaCl₂, 0.04 (pCa = 7); HEPES-KOH, 10; (adjusted to pH = 7.2 with KOH). Electrodes had a resistance of about 5 MΩ. Seal resistances were in the range of 2–5 GΩ.

For recording of calcium currents (I Ca,L, I Ca,S), we replaced K-Aspartate and KCl in the intracellular solution, with an equal amount of CsCl. The extracellular solution contained in (mM/L): tetraethylammonium-chloride (TEA-Cl), 130; CaCl₂, 2; MgCl₂, 1; 4-amino-pyridine, 10; Hepes, 25; (adjusted to pH = 7.4 with TEAOH). I f was recorded in Tyrode, after addition of 10 μM TTX and 0.3 μM isradipine. I f was routinely recorded in Tyrode solution containing 5 mM BaCl₂, to block I f. We performed data acquisition by using the pClamp software (ver. 9, Axon Instruments Inc.).

Data analysis. The AP parameters were calculated as reported previously in references 8, 36 and 37. The following action potential (AP) parameters were calculated: the AP duration (APD), the slopes of the diastolic depolarisation (SDD), the maximum diastolic potential (MDP), the AP threshold (Eth), the maximum upstroke velocity (the peak of the first derivative of the AP waveform dv/dt), and the AP amplitude (APA). The cell membrane capacitance has been monitored by applying brief (10 ms) voltage steps of ±10 mV amplitude from a holding potential (HP) of -35 mV.

The If activation curve was measured according to a protocol described by DiFrancesco and Mangoni and fitted according to the equation where P(V) is the degree of activation of macroscopic If and is equivalent to the voltage dependency of the probability of opening of single f-channels, V 1/2 is the voltage for half activation, and n is the slope factor. Cav-mediated Ca²⁺ currents and I f were analyzed as previously reported in references 20 and 37.

We performed analysis by employing the Origin Lab software (ver. 7.5, Microcal Inc., Northampton, MA USA). Results are presented as means ± the standard error of the mean (SEM, number of cells). For calculating the level of significance, Student’s t-tests, the one- or two-way ANOVA tests followed by Tukey’s post-hoc tests and non-parametric Kruskal-Wallis tests were 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.

Acknowledgements

We thank Elodie Kupfer and the personnel of the IFR3 animal facility of Montpellier for breeding and managing mouse lines. We thank Isabelle Bidaud (CNRS UMR 5203, Montpellier France) for excellent technical assistance. We also thank Stéphanie Barrère-Lemaire (CNRS UMR 5203) and Emilio Carbone (University of Turin, Italy) for helpful discussion.

Financial Support

This work has been supported by CavNet a Research Training Network (RTN) funded through the European Union Research Programme (6FP) MRTN-CT-2006-035367, the INSERM National Program for Cardiovascular Diseases (PNRC), the Fondation de France (Cardiovac 2008002730), the Agence Nationale pour la Recherche (ANR-06-PHISIO-004-01), the NIH R01HL087120-A2 grant, the Deutsche Forschungsgemeinschaft (DFG, IS63/1-1/2) and the Austrian Science Fund (P-20670). Pietro Mesirca and Angelo Torrente are Cα_Net fellows.

Note

Supplementary materials can be found at: www.landesbioscience.com/journals/channels/article/15264
References

1. Booyt MR, Honjo H, Kodama I. The sinoatrial node, a heterogeneous pacemaker structure. Cardiovasc Res 2000; 47:658-87.

2. Molloy FL, Jane MJ. Morphology and electrophysiology of the mammalian atrioventricular node. Physiol Rev 1988; 68:608-47.

3. Efimov IR, Nolinski VP, Rothenberg F, Greener ID, Li J, Dobrzynski H, et al. Structure-function relationship in the AV junction. Annu Rec A Discov Mol Cell Evol Biol 2004; 280:952-65.

4. Hancox JC, Levi AJ. L-type calcium current in rod- and spindle-shaped myocytes isolated from rabbit atrioventricular node. Am J Physiol 1994; 267:1670-80.

5. Munk AA, Adjemian RA, Zhao J, Ogbagbebel A, Shrir A. Electrophysiological properties of morphologically distinct cells isolated from the rabbit atrioventricular node. J Physiol 1996; 493:801-18.

6. Mangoni ME, Nargeot J. Genesis and regulation of the heart automaticity. Physiol Rev 2008; 88:919-82.

7. Honjo H, Booyt MR, Kodama I, Toyama J. Correlation between electrical activity and the size of rabbit sinoatrial node cells. J Physiol 1996; 496:795-808.

8. Lei M, Jones SA, Liu J, Lancaster MK, Fung SS, Dobrzynski H, et al. Requirement of neuronal- and cardiac-type sodium channels for murine sinoatrial node pacemaking. J Physiol 2004; 559:835-48.

9. Horvath K, Buermans HF, Brons JE, Verkerk AO, Bakker ML, Waller V, et al. Gene expression profiling of the forming atrioventricular node using a novel rXb3-based node-specific transgenic reporter. Circ Res 2009; 105:63-9.

10. Dobrzynski H, Nolinski VP, Sambehelshvili AT, Greener ID, Yamamoto M, Booyt MR, et al. Site of origin and molecular substrate of atrioventricular functional rhythm in the rabbit heart. Circ Res 2003; 93:1102-10.

11. Booyt MR, Inada S, Yoo S, Li J, Liu J, Teller J, et al. Connexins in the sinoatrial and atrioventricular nodes. Adv Cardiol 2006; 42:175-97.

12. Miquelot L, Meysen S, Mangoni M, Bois P, van Rijen HV, Abarb P, et al. Architectural and functional asymmetry of the His-Purkinje system of the murine heart. Cardiovasc Res 2004; 63:77-86.

13. Mangoni ME, Nargeot J. Properties of the hyperpolarization-activated current (I) in isolated mouse sinoatrial cells. Cardiovasc Res 2001; 52:51-64.

14. Yuill KH, Hancox JC. Characteristics of single cells isolated from the atrioventricular node of the adult guinea-pig heart. Pflugers Arch 2002; 445:311-20.

15. Papadatos GA, Wellerstein PM, Head CE, Rancliff R, Brady PA, Benndorf K, et al. Slowed conduction and ventricular tachycardia after targeted disruption of the cardiac sodium channel gene Scn5a. Proc Natl Acad Sci USA 2002; 99:6210-5.

16. Pfretzer J, Engel J, Schrott-Fischer A, Stephan K, Bova S, Chen H, et al. Congenital deafness and sinoatrial node dysfunction in mice lacking class D L-type Ca(2+)-currents. Cell 2000; 102:89-97.

17. Lei M, Goldard C, Liu J, Leoni AL, Royer A, Fung SS, et al. Sinus node dysfunction following targeted disruption of the murine cardiac sodium channel gene Scn5a. J Physiol 2005; 567:387-400.

18. Hagihara N, Irisawa H, Kamayama M. Contribution of two types of calcium currents to the pacemaker potentials of rabbit sino-atrial node cells. J Physiol 1988; 395:233-53.

19. Irisawa H, Brown HF, Giles W. Cardiac pacemaking in the sinoatrial node. Physiol Rev 1993; 73:197-227.

20. Mangoni ME, Traboulouse A, Leoni AL, Couette B, Marger L, Le Quang K, et al. Bradycardia and slowing of the atrioventricular conduction in mice lacking Ca3.1/alpha1G T-type calcium channels. Circ Res 2006; 98:1422-30.

21. DiFrancesco D, Mangoni M. Modulation of single hyperpolarization-activated channels (Ih) by cAMP in the rabbit sino-atrial node. J Physiol 1994; 474:87-82.

22. BoSmirchev AI, Briggs J, StarusGG. Inhibitory actions of ZENEGA ZD7288 on whole-cell hyperpolarization activated inward current (Ih) in guinea-pig dissociated sinoatrial node cells. Br J Pharmacol 1993; 110:343-9.

23. Clark RB, Mangoni ME, Lueter A, Couette B, Nargeot J, Giles WR. A rapidly activating delayed rectifier K+ current regulates pacemaker activity in adult mouse sinoatrial node cells. Am J Physiol Heart Circ Physiol 2004; 286:H1757-66.

24. Zhang Q, Timofeyev V, Lu L, Li N, Singapuri A, Long MK, et al. Functional roles of a Ca2+-activated K+ channel in atrioventricular nodes. Circ Res 2008; 102:465-71.

25. Yamamoto M, Dobrzynski H, Teller J, Nowa R, Biller R, Honjo H, et al. Extended atrial conduction system characterised by the expression of the HCN4 channel and connexin45. Cardiovasc Res 2006; 72:721-81.

26. Kodama I, Niknaram MR, Booyt MR, Suzuki R, Honjo H, Owen JM. Regional differences in the role of the Ca2+ and Na+ currents in pacemaker activity in the sinoatrial node. Am J Physiol 1997; 272:H279-306.