TGF-β1-Mediated Fibrosis and Ion Channel Remodeling Are Key Mechanisms in Producing the Sinus Node Dysfunction Associated With SCN5A Deficiency and Aging

Xiaojin Hao, PhD; Yanmin Zhang, PhD; Xinzhao Zhang, BS; Mahesh Nirmalan, PhD; Laura Davies, PhD; Dimitrios Konstantinou, PhD; Fei Yin, BS; Halina Dobrzynski, PhD; Xin Wang, PhD; Andrew Grace, PhD; Henggui Zhang, PhD; Mark Boyett, PhD; Christopher L.-H. Huang, PhD*; Ming Lei, PhD*

Background—Mutations in the cardiac Na⁺ channel gene (SCN5A) can adversely affect electric function in the heart, but effects can be age dependent. We explored the interacting effects of SCN5A disruption and aging on the pathogenesis of sinus node dysfunction in a heterozygous SCN5A knockout (SCN5A⁺/−) mouse model.

Methods and Results—We compared functional, histological, and molecular features in young (3 to 4 month) and old (1 year) wild type and SCN5A⁺/− mice. Both SCN5A disruption and aging were associated with decreased heart rate variability, reduced sinoatrial node automaticity, and slowed sinoatrial conduction. They also led to increased collagen and fibroblast levels and upregulated transforming growth factor-β1 (TGF-β1) and vimentin transcripts, providing measures of fibrosis and reduced Nav1.5 expression. All these effects were most noticeable in old SCN5A⁺/− mice. Na⁺ channel inhibition by Nav1.5-E3 antibody directly increased TGF-β1 production in both cultured human cardiac myocytes and fibroblasts. Finally, aging was associated with downregulation of a wide range of ion channel and related transcripts and, again, was greatest in old SCN5A⁺/− mice. The quantitative results from these studies permitted computer simulations that successfully replicated the observed sinoatrial node phenotypes shown by the different experimental groups.

Conclusions—These results implicate a tissue degeneration triggered by Nav1.5 deficiency manifesting as a TGF-β1-mediated fibrosis accompanied by electric remodeling in the sinus node dysfunction associated with SCN5A disruption or aging. The latter effects interact to produce the most severe phenotype in old SCN5A⁺/− mice. In demonstrating this, our findings suggest a novel regulatory role for Nav1.5 in cellular biological processes in addition to its electrophysiologic function. (Circ Arrhythm Electrophysiol. 2011;4:397-406.)

Key Words: sinoatrial node ■ aging ■ remodeling ■ ion channels ■ Nav1.5 protein

Sinus node dysfunction (SND) is associated with abnormal impulse formation and propagation in the sinoatrial node (SAN). It presents clinically as sinus bradycardia, sinus pause or arrest, atrial chronotropic incompetence, and SAN exit block.² It affects ≈ 1 in 600 cardiac patients aged > 65 years and is responsible for ≈ 50% of the 1 million permanent pacemaker implants per year worldwide.³ Although SND occurs most commonly in elderly patients in the absence of clinically apparent accompanying cardiac disease,² its pathogenesis is unclear. Nevertheless, experimental animal models do show structural, electrophysiological, and ion channel remodeling with age.²,⁴-⁷ Furthermore, genetic defects in ion channels that include those involving human Nav1.5 have been associated with familial sick sinus syndrome, a rare congenital form of SND.⁸-¹⁰ Finally, genetic defects in SCN5A also are associated with progressive (ie, age-dependent) cardiac conduction disease (PCCD), which often is accompanied by SND.¹⁰,¹¹

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The clinical severity of such arrhythmic syndromes among SCN5A mutation carriers varies with genetic background, the presence or absence of molecular modifiers, and particular physiological conditions. Of these factors, age appears to be the most important influence. Thus, slowed conduction in PCCD progressively worsens with age, ultimately leading to
an atrioventricular block requiring pacemaker implantation.\(^1\) Several familial arrhythmic syndromes associated with SCN5A show degenerative fibrotic cardiac abnormalities to extents that vary with age.\(^2\) - \(^4\) but it is uncertain whether this acts through the resulting phenotype or directly participates in the pathogenic process itself.

The recently developed heterozygous Scn5a knockout (Scn5a\(^{+/−}\)) mouse model shows a 50% background haploinsufficiency in the wild-type (WT) Nav1.5 protein.\(^6\) In common with human PCCD,\(^6\) - \(^8\) it shows cardiac conduction slowing with a pattern consistent with age-dependent degenerative cardiac abnormalities. Scn5a\(^{+/−}\) mice also show sinus bradycardia, slowed SAN conduction, and SAN exit block, replicating major features of clinically observed SND in patients.\(^9\) Scn5a\(^{+/−}\) mice also have been reported to display great variability in their electrophysiological phenotype. In fact, there may even be 2 distinct phenotypes—a milder one associated with a delay of myocardial remodeling, aging, and functional Nav1.5 protein.\(^6\) - \(^8\) The present study accordingly uses this model in an investigation of interactions between aging and Scn5a disruption in producing SND and the possible cellular and molecular mechanisms that produce this condition.

Methods

The methods are described briefly herein, with full accounts provided in the online-only Data Supplement.

Animals

All experiments conformed to the UK Animal (Scientific Procedures) Act (1986). Heterozygous Scn5a\(^{+/−}\) mice were generated as previously described.\(^6\) Four groups of mice were used: young WT and young Scn5a\(^{+/−}\) mice (aged 3 to 4 months) and old WT and old Scn5a\(^{+/−}\) mice (aged 12 months, equivalent to a 3-year-old rat and a 60- to 65-year-old human).

Electrophysiological Studies in Anesthetized Animals

Surface ECG recordings were made using a Powerlab 26T system (ADInstruments; Oxford, UK) and Chart version 6.0. Corrected SAN recovery time was measured by an esophageal pacing protocol. Heart rate variability (HRV) was examined and presented as Poincare plots.

Electrophysiological Studies in Isolated SAN Tissue Preparations

Multielectrode array recordings of extracellular potentials (online-only Data Supplement Figure 1) applied to isolated SAN preparations permitted construction of SAN activation maps and measurements of SAN cycle length (CL) and sinoatrial conduction time (SACT) as described previously.\(^6\)

Histology and Immunohistochemistry

Selected SAN regions were first identified by anti-HCN4 immunostaining. The adjacent sections were used for either picrosirius red collagen staining or vimentin fibroblast immunostaining.

RNA Isolation and Real-Time Polymerase Chain Reaction

Total RNA was extracted from frozen SANs using an RNasy Micro Kit (Qiagen), and cDNA was synthesized using the Superscript III First Strand Synthesis System (Invitrogen). Random hexamers were chosen as the primer. Real-time polymerase chain reaction was performed using either the Taqman or SYBR green systems (Applied Biosystems). The mRNA levels of each gene were normalized to HPRT levels.

Nav1.5-E3 Antibody Treatment of Human Neonatal Cardiac Myocytes and Fibroblasts

Transforming growth factor-β (TGF-β) was detected at transcriptional and protein levels in cultured human neonatal cardiac myocytes and fibroblasts after 24 hours of treatment with Nav1.5-E3 antibody.\(^6\)

Patch Clamping of Cultured Human Neonatal Cardiac Myocytes

Fast inward Na current (\(i_{\text{Na}}\)) was recorded by voltage clamping to compare cells with and without treatment of Nav1.5-E3 antibody for 24 hours.

Computer Simulations

Computer simulations of electrophysiological function in SAN cells used RNA levels of the individual ion channels in the different groups derived from real-time polymerase chain reaction (online-only Data Supplement Table 1). They simulated the pacemaking CLs of the SAN cells and the conduction times between the SAN and the atrium (measured as the time delay between the excitation of the SAN and the atrial cells). The starting point was a recently developed mathematical mouse SAN cell model.\(^6\)

Statistical Analysis

The experiments were designed to examine both the main effect of the 2 factors Nav1.5 deficiency and age as an outcome that shows consistent differences between levels of a factor (age or Nav1.5 deficiency) and the interaction between these 2 factors; namely, an interaction effect is said to exist when differences in 1 factor depend on the level of the other factor. These effects were analyzed by a 2-way factorial ANOVA, namely, the effects of Scn5a disruption (WT versus Scn5a\(^{+/−}\)), aging (young versus old), and interactions between these. All values are presented as mean±SEM. Student \(t\) test was used in experiments involving Nav1.5-E3 antibody treatment on human neonatal cardiac myocytes and fibroblasts. Nonlinear and linear regression studies were performed to investigate correlations between parameters. \(P<0.05\) was considered to indicate significant differences.

Results

Electrocardiographic Characteristics

SAN automaticity was first characterized through electrocardiographic recordings and esophageal electric pacing in anesthetized mice. Figure 1 shows typical electrocardiographic recordings illustrating slower heart rates in Scn5a\(^{+/−}\) compared with WT mice (Figure 1A, top 2 traces). The occasional young (2 of 11) and old Scn5a\(^{+/−}\) (1 of 10) mice, but none of the young (0 of 19) or old WT (0 of 10) mice, showed more extreme manifestations of SAN malfunction in the form of sinus pause/arrrest or atrioventricular conduction block (Figure 1A, bottom 2 traces). Online-only Data Supplement Table 2 correlates longer P-wave durations and longer PR, QRS, and QTc intervals with Scn5a disruption but not age. Corrected SAN recovery time after esophageal pacing also increased with the Scn5a disruption (Figure 1B and 1C, online-only Data Supplement Table 2) but not with age. Nevertheless, Poincaré plots between each RR interval and its immediately preceding RR interval showed reduced scatter with both aging and Scn5a disruption, with old Scn5a\(^{+/−}\) mice producing the tightest cluster (Figure 2A). Such findings suggest a decreased HRV consistent with...
Figure 1. Characteristics of surface electrocardiographic recordings and CSNRT obtained by esophageal pacing in anesthetized mice. A, Representative recordings of surface ECG. Compared to WT mice, Scn5a+/− mice showed prolonged RR interval, sinus pause, and AV block. B, Representative SNRT from WT and Scn5a+/− mice. C, CSNRT in young WT (n=19), young Scn5a+/− (n=11), old WT (n=10), and old Scn5a+/− (n=10) mice. Two-way ANOVA P=0.002 for Scn5a+/− versus WT mice. AV indicates atrioventricular; CSNRT, corrected sinoatrial node recovery time; O, old mice; WT, wild type; Y, young mice.

alterations in autonomic modulation. These findings were quantified as the standard deviation of the change in RR intervals obtained from the differences between consecutive pairs of RR intervals, revealing significant differences in both Scn5a disruption and aging and the greatest reduction in the combined condition (Figure 2B).

Mapping of Impulse Generation and Conduction in Isolated SAN Preparations

The detailed intrinsic electric properties of the SAN were then characterized by multielectrode array recordings of extracellular potentials from SAN preparations (online-only Data Supplement Figure 1) isolated from their autonomic inputs. Activation at each recording site gave detectable, often biphasic, electrogram deflections from which intrinsic CL and SACT can be determined. The primary pacemaker region was determined by the earliest deflection in the array (starred traces). The left side of Figure 3A shows typical results from the different experimental groups. Analysis of the latencies of these electric deflections permitted construction of isochronic activation maps (Figure 3A, right).

Quantitative analysis (Figure 3B and 3C) indicates that both aging and Scn5a disruption significantly prolonged both intrinsic CL (young WT, 164±8 ms [n=5]; young

Scn5a+/−, 211±12 ms [n=7]; old WT, 231±25 ms [n=8]; old Scn5a+/−, 321±30 ms [n=7]) and SACT (young WT, 8.0±0.5 [n=5]; young Scn5a+/−, 11.4±0.7 [n=7]; old WT, 12.0±1.1 ms [n=8]; old Scn5a+/−, 21.9±1.9 [n=7]). Furthermore, these effects interacted to produce the greatest functional changes in the old Scn5a+/− hearts (CL Scn5a+/− versus WT, P=0.005; CL old versus young, P<0.001; SACT Scn5a+/− versus WT, P<0.001; SACT old versus young, P<0.001; interaction of the 2 effects, P=0.04). Thus, there was a decreased SAN automaticity and slower conduction (within SAN and from SAN to surrounding atrium) in Scn5a+/− compared to WT mice and in old compared to young mice, with the old Scn5a+/− mice showing the slowest automaticity and the slowest conduction.

Interstitial Fibrosis in the SAN

To determine whether these physiological changes associated with aging and Scn5a disruption were accompanied by degenerative structural abnormalities in the SAN, we examined their corresponding abundances of collagen and fibroblasts, the main interstitial components. The SAN regions selected for the study were identified by HCN4 staining (online-only Data Supplement Figure 2). The adjacent sec-
tions then were used for either picrosirius red or vimentin immunostaining. The picrosirius red staining associated both aging and Scn5a disruption with increased collagen abundance (dark red) in the SAN area (Scn5a/H11001/H11002 versus WT, \( P < 0.002 \); old versus young, \( P < 0.04 \)) (Figure 4A and 4C). Vimentin immunostaining then demonstrated parallel increases in fibroblast density (green) in the SAN area (Scn5a/H11001/H11002 versus WT, \( P < 0.017 \); old versus young, \( P < 0.001 \)) (Figure 4B and 4D). In both cases, the greatest effects occurred in the old Scn5a/H11001/H11002 hearts.

Alterations in Transcripts of Fibrotic Regulatory Genes

These tissue fibrotic changes were accompanied by alterations in fibrotic regulatory transcripts. Aging and Scn5a disruption both upregulated transcription of the major fibrosis cytokine TGF-\( \beta_1 \) and the fibroblast marker vimentin in the SAN. Again, the greatest effects were in the old Scn5a/H11001/H11002 mice (TGF-\( \beta_1 \), Scn5a/H11001/H11002 versus WT, \( P < 0.005 \); TGF-\( \beta_1 \) old versus young, \( P < 0.005 \); interaction between the 2 effects, \( P < 0.02 \); vimentin Scn5a/H11001/H11002 versus WT, \( P < 0.008 \); vimentin old versus young, \( P < 0.001 \); interaction between the 2 effects, \( P < 0.003 \)) (Figure 5A). Both aging and Scn5a disruption were associated with a significant downregulation of Nav1.5 expression (Scn5a/H11001/H11002 versus WT, \( P < 0.02 \); old versus young, \( P < 0.005 \)) (Figure 5A) to give the following sequence of falling Nav1.5 expression: young WT > young Scn5a/H11001/H11002 > old WT > old Scn5a/H11001/H11002. There was a surprisingly significant nonlinear negative regression between both TGF-\( \beta_1 \) and vimentin and Nav1.5 expression (TGF-\( \beta_1 \), \( R^2 < 0.77 \), \( P < 0.001 \); vimentin, \( R^2 = 0.78 \), \( P < 0.001 \)) (Figure 5B) as well as a less surprising linear correlation between vimentin and TGF-\( \beta_1 \) expression (\( R^2 = 0.85 \), \( P < 0.001 \)) (Figure 5B).

The surprising correlation between Nav1.5 and fibrotic genes then prompted direct tests of the hypothesis that reduced Nav1.5 channel expression or activity might acutely upregulate TGF-\( \beta_1 \) and vimentin transcripts in cardiac myocytes and cardiac fibroblasts, both known to express...
Nav1.5.21 Twenty-four-hour incubations with 15 μg/mL Nav1.5-E3 antibody, known specifically to inhibit Nav1.5 protein in the cell culture medium (antibody treatment versus control for either myocytes or fibroblasts, P=0.04 for transcripts of myocytes, P=0.03 for transcripts of fibroblasts, and P<0.001 for protein) (Figure 5C). As shown in online-only data Supplement Figure 3, such increased TGF-β1 mRNA/active form TGF-β1 protein in myocytes treated with Nav1.5-E3 antibody is associated with an ≈20% reduction in Nav1.5 protein expression and an ≈50% reduction in peak iNa current density compared to nontreated cells. Nav1.5 mRNA expression in these myocytes, however, remained unchanged (data not shown).

Remodeling in Transcription of Ion Channel Genes
We then investigated the effects of either aging or Scn5a disruption on transcriptional levels of 85 ion channel and regulatory genes in the SAN. Figure 6 and online-only data Supplement Table 3 list the genes showing statistically significant changes in relationship to aging and Scn5a disruption. These demonstrate that Nav1.5 was downregulated with both the Scn5a+/− and the aging conditions (Figure 6A). In contrast, there were 220% and 57% increases in Nav1.1 expression in young and old Scn5a+/− mice, respectively, compared with young WT mice (Scn5a+/− versus WT, P=0.03) (Figure 6A), consistent with a compensation for the deficiency in Nav1.5.

Figure 6 shows that 33 transcripts for all the remaining major voltage-gated K+ and Ca2+ channels, connexins, and Ca2+-handling transcripts also were downregulated with age in both WT and Scn5a+/− mice. These were:

1. Navβ3, 1 of the Na+ channel regulatory subunits (Figure 6A and 6B)
2. HCN1 and HCN2 but not the major SAN HCN subunit HCN4 (Figure 6C)
3. Sixteen of 38 K+ channel transcripts, including Kv1.2, Kv1.4, Kv1.5, Kv1.6, Kv4.2, KvLQT1, SK1, Kvβ1, MiRP3, MiRP4, Kir3.1, Kir3.4, Kir6.1, Kir6.2, Meag, and Kvβ2 (Figure 6D and 6E)
4. Six of 21 Ca2+ channel and Ca2+-handling protein transcripts, including Cav1.3, Cavβ3, Cav-α281, NCX1, Calm3, and RIP3-2 (Figure 6F) (Transcription of a major ion channel for generating the SAN pacemaker potential, Cav1.3, was decreased by 55% and 73% in old WT and old Scn5a+/− mice, respectively, compared to young WT mice. Transcription of NCX1, responsible for the Na+-Ca2+ exchanger, was decreased by 49% and 72% in old WT and Scn5a+/− mice, respectively.)
5. CLC3, Cfrt, Trpc3, and ATP1a1, among other ion channels and transporters (Figure 6G)
6. Gap junction channels where there was decreased expression of Cx40 but not of Cx43 and Cx45, the latter a major SAN gap junction channel (Figure 6G)
7. Tbx3, an identified crucial transcriptional factor controlling ion channel gene expression program and phenotype22 in the SAN (Figure 6H).

Taking all ion channels into account, there was a significant decrease in overall ion channel gene expression related to aging (P<0.001) (Figure 6I). Overall, the abundance of ion channel gene expression in the young Scn5a+/− mice tended to be greater than in young WT mice, whereas in the aged mice, the overall channel gene expression of old Scn5a+/−mice tended to be lower than in young WT mice, whereas in the aged mice, the overall channel gene expression of old Scn5a+/−mice tended to be lower than in young WT mice.
mice was lower than in old WT mice. Furthermore, there was a significant interaction between the aging and Scn5a disruption, and ion channel expression generally was lowest in the old Scn5a<sup>−/−</sup> mice (P<0.001) (Figure 6I).

Finally, there was an upregulation of expression of the β<sub>1</sub>-adenergic receptor, central to cardiac adrenergic signaling, associated with Scn5a disruption (Scn5a<sup>−/−</sup> versus WT, P=0.04) (Figure 6J). However, neither aging nor Scn5a disruption affected β<sub>1</sub>-adenergic receptor or muscarinic M<sub>2</sub>-receptor transcripts.

### Computer Simulations

The experimental results associated aging and Scn5a disruption not only with tissue degeneration (featured as TGF-β<sub>1</sub>-mediated fibrosis), but also with transcriptional alterations in a large range of ion channels at the mRNA level. Our final investigations explored the implications of these changes for cell excitation and its propagation between cells and the extent to which this would account for the experimental observations, and so, they provide possible underlying mechanisms for the phenomena observed.

First, SAN action potentials in young WT mice were simulated using a previously developed action potential model of a mouse SAN myocyte from Kharche et al<sup>20</sup> (Figure 7A). This model then led to similar simulations for the old mice (Figure 7A and 7B). They similarly replicated experimental findings that the old mice showed the slowest pacemaker activity (Figure 7A and 7B).

Second, the atrial SAN cell coupling model successfully replicated the observed slowing of SAN conduction with aging and Scn5a disruption. Thus, explorations of the effect of reducing the junctional conductance g<sub>j</sub> between cells (Figure 7C) further demonstrated prolongations of conduction times most obvious in the old Scn5a<sup>−/−</sup> mice. Decreases of g<sub>j</sub> to ~1.2 nanosiemens replicated the experimental conduction pattern in which SAN-atrial conduction was slowed in the old WT and young Scn5a<sup>−/−</sup> conditions compared to young WT mice, with the greatest slowing in old Scn5a<sup>−/−</sup> mice (Figure 7C and 2D).

### Discussion

The present studies explored the relationships between the pathogenesis of SND and aging and Scn5a disruption as well as the possible interactions between these factors using the Scn5a<sup>−/−</sup> mouse model known to directly replicate Scn5a haploinsufficiency.<sup>14</sup> Our results suggest that both electric
remodeling and tissue degeneration, detected as TGF-β1-mediated fibrosis, affect pacemaker and conduction function in SND associated with Scn5a disruption or aging, with a combination of both aging and Scn5a disruption producing the most severe phenotype. In implicating Nav1.5 deficiency in such changes, we also suggest a novel regulatory role for Nav1.5 in cellular biological processes extending beyond its electrophysiologic function. Finally, the modeling studies at least partially reconstructed the physiological mechanisms by which both aging and Scn5a disruption lead to SND, thereby...
drawing parallels between these and similar conduction changes in the ventricle that occur in the possibly related condition of PCCD.

Our physiological studies first demonstrated that aging and Scn5a disruption affected in vivo heart electrophysiological properties. Thus, Scn5a disruption significantly increased P-wave durations; RR, PR, and QRS intervals; and SAN recovery times. Aging did not exert effects in such in vivo recordings in intact animals, which could be due to autonomic alterations known to increase sympathetic activation with age as has been observed in humans.23,24

Interacting effects of aging and Scn5a disruption were observed in Poincaré plots representing HRV (Figure 2). Such HRV alterations are considered critically predictive of cardiac arrhythmic events in the form of ventricular tachycardia and sudden cardiac death.25,26 In live animals, HRV results from cyclic inputs from the autonomic nerves acting on the SAN. Consistent with previous studies,27 we demonstrated that HRV decreases with aging. We also associate for the first time to our knowledge decreases in HRV with Scn5a disruption. Scn5a gene disruption but not aging led to a marked upregulation of β1-adenergic receptor transcript levels, further implicating alterations in autonomic function associated with the Scn5a gene disruption.28 However, the Nav1.5 downregulation also might be involved in both aging- and Scn5a gene disruption-related HRV alteration. Therefore, the greatest decrease in HRV was observed in old Scn5a+/-/- mice, which showed the lowest Nav1.5 expression.

Our results indicate that both aging and Scn5a disruption affected the intrinsic electrophysiological properties of the SAN itself. Thus, they exerted both individual and interacting effects in increasing CL and SACT in ex vivo SAN preparations isolated from their autonomic inputs. These alterations in turn correlated with corresponding alterations in fibrotic processes and ion channel and regulatory gene transcriptional remodeling.

Of these, the alterations in fibrotic processes directly parallel previous reports demonstrating an association between the Scn5a+/-/- condition and ventricular fibrotic changes15,16 as well as a link between age-dependent SAN dysfunction and fibrosis,29 although such features were not observed in all studies.5,30 Our present findings associated both Scn5a disruption and aging with fibrosis in the SAN. Thus, they demonstrated significant positive interacting effects of Scn5a disruption and age on collagen and fibroblast levels, with the greatest effects seen in the old Scn5a+/-/- hearts. The resulting fibrosis could potentially slow conduction both within the SAN and from the SAN to the surrounding atrium. Aging and Scn5a disruption correspondingly resulted in interacting upregulatory effects on levels of the key modulator of fibrosis TGF-β1 and the fibroblast marker vimentin, similarly showing maximal effects in the old Scn5a+/-/- hearts. Altered expression of TGF-β1 and vimentin transcripts is associated with increased collagen and fibroblast abundance, indicating that interstitial fibrosis occurred. Further experiments implicated Nav1.5 deficiency in the underlying mechanism for these findings and provided a possible causal link between Nav1.5 deficiency and increased fibrosis in the SAN. Both Scn5a disruption and aging exerted strong downregulatory effects on Nav1.5 expression levels, giving the following order: old Scn5a+/-/-<old WT<young Scn5a+/-/-<young WT. This finding provided a range of Nav1.5 expression levels against which measurements of TGF-β1 and vimentin expression levels could be compared. The latter revealed strong negative correlations between the expressions of TGF-β1 and vimentin and the expression of Nav1.5. Further in vitro experiments indicated that Na+ channel inhibition by either Nav1.5-E3 antibody or Na channel blocker significantly increased TGF-β1 production by both cardiac myocytes and cardiac fibroblasts; previous studies have reported that both cell types normally express Nav1.5 and secrete TGF-β1.31 Although the detailed mechanistic link needs further investigation, our results suggest that at least 2 potential mechanisms may underlie the Nav1.5-deficiency-associated TGF-β1-mediated SAN fibrosis. First, TGF-β1-dependent signaling can be regulated by Na channel activity. Our results show that increased TGF-β1 mRNA/active form TGF-β1 protein in Nav1.5-E3 antibody-treated myocytes is associated with a significant reduction (50%) in 1Na current density (online-only Data Supplement Figure 3). Such a result is consistent with the previous study by Ugarte and Brandan32 in rat primary myocytes. The authors showed that the inhibition of electric activity by Na channel blocker upregulated TGF-β activity. In contrast, the promotion of electrophysiological activity in myotube cultures induced by the upregulation of voltage-dependent Na channels or by direct stimulation with extracellular electrodes downregulated TGF-β activity in rat primary myotubes. Similar results also were obtained in denervated adult muscles.33 The altered 1Na channel activity may affect the intracellular environment, such as pH, which has been shown to activate TGF-β signaling.

Second, through its interacting proteins, Nav1.5 deficiency leads to activation of TGF-β1 signaling activation, which is consistent with suggestions that Nav1.5 forms multiprotein complexes with Nav1.5-interacting proteins34,35 through which Nav1.5 may exert a regulatory role in cellular biological processes beyond its electrophysiologic function. The decreased Nav1.5 channel expression (online-only Data Supplement Figure 3) or altered configuration of channel complex could potentially underlie such mechanisms. Thus, the present findings should promote further investigations into the causal relationship between Nav1.5 channel deficiency and activation of TGF-β1 signaling.

The second major finding bearing on the mechanisms underlying the electrophysiological changes concerns alterations in expression of a wide range of ion channels and regulatory genes in the SAN associated with aging and Scn5a disruption. There was reduced Nav1.5 expression not only with Scn5a disruption, but also with age. A further reduction in Nav1.5 in the SANs of aged mice indicates that aging impairs Nav1.5 expression in the SAN as an independent factor. Such an effect may account for the phenotypic variability of Nav1.5-defect-associated SND. In a recent study reported by Yanni et al.,7 a lack of the expression of Nav1.5 in old rat SAN manifested in a significant decline in sinus rate and slow sinoatrial conduction. Such age-related reduction in expression of Nav1.5 could help to explain the
increase in the SAN conduction time observed in elderly patients and SAN exit block in sick sinus syndrome. In addition, aging but not the Scn5a disruption produced a wide range of downregulatory effects on ion channels. These findings took place along with a reduction in expression of the crucial transcription factor Tbx3 that is known to regulate the pacemaker gene expression program in the SAN, suggesting a mechanism for the observed ion channel downregulation in the SAN.

Some of the additional ion channel changes also could be related to the observed physiological changes. Thus, downregulation of the regulatory Na\(^+\) channel subunit Navβ3 (≈70%) could affect the SND phenotype. The observed downregulation of Ca\(^{2+}\) channel and Ca\(^{2+}\)-handling protein transcripts (Figure 6F) was consistent with previous reports of age-dependent t-type Ca\(^{2+}\) channel deficiencies in the SAN correlating with SND in aged guinea pig hearts. The downregulation of key Kv4.2, KvLQT1, Meag, and Kv1.5 K\(^+\) channel transcripts would be expected to prolong pacemaker action potential repolarization and potentially contribute to sinus bradycardia or arrhythmias in SND. Finally, reduced Cx40, but not Cx43 and Cx45, together with decreased Na\(^+\) current and fibrosis, could slow conduction within the SAN and from SAN to the surrounding atrium. Our computer simulations indeed demonstrated that the ion channel remodeling resulted in slowed pacemaker activity and increased the SAN-atrium conduction time after aging and Scn5a disruption and, thereby, could contribute to a mechanism for our observations.

In conclusion, the present study demonstrates for the first time to our knowledge that both aging and Scn5a disruption contribute to the pathogenesis and severity of SND through mechanisms involving TGF-β-mediated fibrosis associated with Na\(^+\) channel deficiency accompanied by transcriptional remodeling of ion channels. These findings also have important implications for possible mechanisms underlying other arrhythmic syndromes associated with Nav1.5 haploinsufficiency, such as PCCD and Brugada syndrome. These also are associated with degenerative fibrotic cardiac abnormalities that depend on age. The present findings, therefore, will prompt future investigations in the Scn5a and other model systems further investigating the mechanisms underlying the alterations in the fibrotic regulatory pathway suggested here as well as the extent to which these are specific to the SAN or can be generalized to myocardial or conduction system components in the rest of the heart. Such clarifications will have important implications in their application to the development of future management strategies for these disease conditions.

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Disclosures

None.

References


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**CLINICAL PERSPECTIVE**

Sick sinus syndrome (also called sinus node dysfunction [SND]) refers to the abnormalities in sinus node impulse formation and propagation phenotypically manifesting as sinus bradycardia, sinus pause/arrest, chronotropic incompetence, sinoatrial exit block, and atrial tachyarrhythmias. It affects ≈1 in 600 cardiac patients aged >65 years and is responsible for ≈50% of the 1 million permanent pacemaker implants per year worldwide. The pathogenesis of SND and its relationship to the aging process are poorly understood. The present studies explored relationships among the pathogenesis of SND, aging, and *Scn5a* disruption as well as the possible interactions among them using the *Scn5a*–/– mouse cardiac model known to directly replicate *Scn5a* haploinsufficiency. They implicate both electrophysiologic remodeling and tissue degeneration, detected as a transforming growth factor-β1-mediated fibrosis, in the altered pacemaker and conduction function in SND associated with *Scn5a* disruption or aging, with a combined presence of both factors producing the most severe phenotype. In implicating Nav1.5 deficiency in these changes, we suggest novel regulatory roles for Nav1.5 in biological processes extending beyond its electrophysiologic function. Finally, modeling studies partially reconstructed possible physiological mechanisms by which both aging and *Scn5a* disruption lead to SND. It points out parallels between these and similar ventricular conduction changes occurring in the potentially related progressive cardiac conduction defect. These results thus provide a basis for further studies seeking understanding of the pathogenesis of aging and *SCN5A*-deficiency-associated heart diseases and may contribute to the development of future therapies for their management.
TGF-β1-Mediated Fibrosis and Ion Channel Remodeling Are Key Mechanisms in Producing the Sinus Node Dysfunction Associated With SCN5A Deficiency and Aging
Xiaojin Hao, Yanmin Zhang, Xinzhao Zhang, Mahesh Nirmalan, Laura Davies, Dimitrios Konstantinou, Fei Yin, Halina Dobrzynski, Xin Wang, Andrew Grace, Henggui Zhang, Mark Boyett, Christopher L.-H. Huang and Ming Lei

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SUPPLEMENTAL MATERIEL

Extended Methods

Animals

Mice with heterozygous targeted knockout of Scn5a gene (Scn5a+/−) were generated and inbred onto a 129/Sv genetic background as described previously. Unless otherwise stated, all experiments grouped equal numbers of male and female Scn5a+/− and WT littermates. The 4 groups of mice studied consisted of young wild type (WT) and young Scn5a+/− mice (3-4 months of age), and old WT and old Scn5a+/− mice (age ≥12 months). All animal procedures conformed with the United Kingdom Animals (Scientific Procedures) Act 1986.

Electrophysiological studies in anaesthetized animals

Mice were anaesthetised with intraperitoneal injection of 2,2,2-Tribromoethanol (Sigma-Aldrich, Cambridge, UK) (250 mg/kg). Three-lead electrocardiogram (ECG) was recorded using subcutaneous electrodes with connection to Powerlab 26T system (AD Instruments, Hasting). The digital recording (16 bit, 1 kHz/channel) at band pass filter settings of 0.5 to 500 Hz (ECG recording) or 2 to 200 Hz (oesophageal ECG recordings) using the Chart v6.0 program (Chart™ 5 Pro) (AD Instruments, Hastings, UK). The ECG measurements provided RR intervals, P wave durations, PR intervals and QRS durations. Sinus node recovery time (SNRT) was assessed after pacing trains of oesophageal stimulation (cycle length of 100 ms, duration 30 s through an ultra-miniature electrophysiological catheter inserted into the oesophagus (ERP-800 Electrophysiology Catheter, Millar Instruments, USA). SNRT was calculated by ECG as the duration between the last stimulus artifact to the next earliest activity, then corrected to the underlying cycle length to give the CSNRT value. Heart rate variability (HRV) was assessed from baseline ECG recordings covering at least 500 consecutive RR intervals, and represented by Poincaré plots of the duration of each normalised (normalised by mean CL) RR interval (RRn) against that of its immediately preceding normalised RR interval (RRn+1). The variability was then quantified by standard derivation of (SD) of delta RR intervals derived from the each normalised RR interval subtracted by that of the preceding normalised RR interval.

Electrophysiological studies of isolated sino-atrial preparations

Extracellular potential (ECP) recordings using a custom-made 64 multi-electrode array or modified bipolar electrodes monitored both sinoatrial node (SAN) automaticity and conduction in isolated sino-atrial preparations. The sino-atrial tissue preparation was set up as described previously (Figure S1). In brief, after dissection of the SAN and surrounding atrial muscle, the preparation was placed in a tissue bath with endocardial surface facing upwards. The preparation was superfused with Tyrode’s solution in 37°C at a rate of 4–5 ml/min. First, multi-electrode array was placed on the SAN surface. These provided ECP recordings over 10 second recording periods performed in triplicate in each SAN for subsequent off-line analysis. The latter derived SAN activation maps using bespoke software that analysed the observed ECPs. Secondly, the bipolar electrode recordings used one pair of reference electrodes positioned on the atrial muscle near the crista terminalis and exploring electrodes successively placed on different points of the SAN. The time intervals between electrophysiological deflections at the exploring and reference electrodes were then determined. The site showing the earliest SAN activation was determined. Intrinsic SAN beats and the sino-atrial conduction time (SACT) were measured from recordings obtained at this site. Electrical signals were digitized at 5 kHz by a DigiData 1322A analog to digital converter (Axon Instruments Inc.) and stored on a computer for later analysis.

Electrophysiological studies of single cells

Cells were cultured and kept in 37°C 5% CO₂ incubator for overnight before doing whole cell patch clamp and the cover slips were taken out of the petri-dish and transferred into the chamber on
microscope. Whole-cell configuration of the voltage patch-clamp technique was used to record $I_{\text{Na}}$. Experiments were performed at room temperature (22–23°C). The electrophysiological characteristics were recorded by Axon 200B amplifier (Axon Instrument). The electrodes were made with borosilicate glass capillaries (1.5mm O.D. X 1.17mm I.D., Harvard Apparatus, Kent, UK) by gravity puller (Model PP-830, Narishige, Japan) with resistance range of 1.0-1.5MΩ with filled internal solution (in mmol/L, KCl 10, CsCl 130, NaCl 10, HEPES 10, pH=7.2 with CsOH). External recording solution are: (in mmol/L: NaCl, 10; NMDG-Cl, 120; CaCl$_2$, 2; MgCl$_2$, 1.2; CsCl, 5; HEPES, 10; Glucose 10 (pH was adjusted to 7.4 with CsOH). Holding potentials were $-120$ mV and $I_{\text{Na}}$ densities (pA/pF) were obtained by dividing the peak $I_{\text{Na}}$ by the cell capacitance. Peak currents were measured during a voltage-clamp protocol.

RNA isolation and real-time PCR

**RNA isolation and reverse transcription.** SANs were dissected from mice following cervical dislocation and frozen under -80°C, then homogenised with Lysing Matrix Beads (MP Biomedicals, Cambridge, UK) using Hybaid Ribolysor (Hybaid, Middlesex, UK). RNA isolation was performed with RNeasy Micro Kit (Qiagen, Fleming Way, Crawley, West Sussex, UK) following manufacturer’s instructions. Total RNA was quantified with Nanodrop ND-1000 Spectrophotographer (Labtech International Ltd, Ringmer, East Sussex, UK) and the quality accessed using RNA 6000 Naco kits run in an Agilent 2100 Bioanalyzer system (Agilent Technologies, South Queensferry, West Lothian, UK). Only samples with an RNA integrity number above 7 were used in the following experiments. Total RNA was subsequently transcribed into cDNA with superscript® III First Strand Synthesis System (Invitrogen, Paisley, UK). Random hexamers was chosen as primers.

**Taqman real-time PCR.** Gene expression levels in the SAN were detected with Taqman low density micro fluidic cards with preloaded primers and probes (Applied Biosystems, Warrington, United Kingdom). The following were examined: 7 genes for sodium channel subunits (Nav1.1, Nav1.3, Nav1.4, Nav1.5, Nav1.7, Navβ1, Navβ3), 21 genes for calcium channel subunits and calcium handling proteins (Cav1.1, Cav1.2, Cav1.3, Cav3.1, Cav3.2, Cavβ1, Cavβ2, Cavβ3, Cava2δ1, Cava2δ2, Cava2δ3, Cavγ4, NCX1, RYR2, RYR3, Calm1, Calm3, Casq2, Pln, RIPC2-3, SERCA2A), 38 genes for potassium channel subunits (Kv1.1, Kv1.2, Kv1.3, Kv1.4, Kv1.5, Kv1.6, Kv2.1, Kv4.1, Kv4.2, Kv4.3, KvLQT1, Merg, meag, SK1, SK2, SK3, Kvβ1, Kvβ2, Kvβ3, KChIP1, KChIP2, Mink, MiRP1, MiRP2, MiRP3, MiRP4, Kir1.1, Kir2.1, Kir2.2, Kir3.1, Kir3.4, Kir3.2, Kir6.1, Kir6.2, TWIK1, TREK1, TRAAK, TASK2), 3 for hyperpolarisation-activated cyclic nucleotide-gated potassium channels (HCN1, HCN2, HCN3), 4 for gap junction proteins (Cx43, Cx37, Cx40, Cx30.2), 2 for transcription factor (Tbx2, Tbx3) and other genes (Clcn2, Clcn3, Cfr, Na, K-ATPase, Trpc3, Caveolin 3). Hprt was used as an endogenous control. All gene expression was quantified by the ABI Prism® 7900HT sequence detection system (Applied Biosystems). The experiment was performed using the Taqman One Step RT PCR Master mix (Applied Biosystems, Warrington, UK) and 100 ng of cDNA. The thermal cycling conditions comprised 2 min at 50°C, 10 min at 94.5°C and 40 cycles of 15 sec, denaturation at 97°C and 60 sec annealing at 59.7°C. Each gene was analyzed using the $2^{-\Delta\Delta C_t}$ method. All the expression data among the four experimental groups were normalized to mean values obtained from the young WT mice.

**Sybr Green real-time PCR** TGF-β₁, vimentin, Cx45, β₁-adrenergic receptor, β₂-adrenergic receptor, muscarinic M2 receptor, HCN4 and ANP gene expression were detected with Sybr Green. Experiments were performed using the Power SYBR® Green PCR Master Mix (Applied Biosystems, Warrington, UK) and primers (Qiagen, Fleming Way, UK). The cycling conditions included a hot start at 95°C for 5 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Normalization and data presenting and analysis were preformed with the same method as Taqman real-time PCR as described above.

To minimize contamination from atrial material in the SAN samples, only samples with relative abundance of ANP and HCN4 ratio less than 30% (indication of atrium contamination) were used in the subsequent real time PCR experiments.
Histology and immunohistochemistry

Isolated SANs were embedded in OCT, snap frozen in liquid nitrogen and stored at -80°C. The SAN was then sectioned continuously in 5 μm slices and the SAN area distinguished by immunohistochemistry for HCN4 positive staining (Figure S2). Sections were incubated with rabbit anti-HCN4 polyclonal IgG (1:100; Alomone Lab, Jerusalem, UK) followed by secondary antibody goat anti-rabbit IgG, FITC conjugated (1:200; Millipore, Hatters Lane, UK). The sections slides adjacent to those showing HCN4-positive staining were chosen for collagen and fibroblast detection. Collagen content was evaluated with picrosirius red (Sigma-Aldrich, Cambridge, UK) staining, visualized using a DM5000B light microscope (Leica, Konwilhill, UK). Fibroblast cells were detected by incubation with guinea pig anti-vimentin (1:100; Progen, UK) followed by donkey anti-guinea pig IgG staining (1:200; FITC conjugated, Chemicon, UK). Slides were double stained using Caveolin 3 to distinguish cardiac myocytes from fibroblasts (first antibody: mouse anti-Caveolin 3, 1:100, AlomoneLab, UK; second antibody: donkey-anti mouse IgG, 1:200, Cy3-conjugated, Chemicon, UK). Florescent images were then acquired using a confocal laser scanning microscope (LSM 510; Zeiss, Germany) equipped with argon and helium–neon lasers, which excited at wavelengths of 488 and 568 nm for detection of fluorescein isothiocyanate (FITC) and/or Cy3, respectively. Following acquisition of images of picrosirius red and fibroblast positively staining areas in SAN were quantified using the image analysis program Velocity (Improvisation, UK) as percentages of field of view for the different groups of mice.

TGF-β detection after Nav1.5-E3 antibody treatment on human neonatal cardiac myocytes and fibroblasts

Human neonatal cardiac myocytes and human neonatal fibroblasts purchased from the HSC cell network (UK) were cultured separately in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS). Cells were then seeded on 24-well plates at densities of 5 X10^4 and 2.5 X 10^4 cells per well for cardiac myocytes and cardiac fibroblasts, respectively. The Nav1.5 specific antibody Nav1.5-E3 6 was applied to 24-well plates at a concentration of 15 μg/ml in 2.5% FCS in the culture medium one day after seeding. This concentration was based on a previous study reporting successful functional inhibition of Nav1.5 channels under such conditions.6 Cardiac myocytes and fibroblasts were harvested for extraction of total RNA 24 hours after antibody treatment. Total RNA was extracted using the Qiagen mini kit following manufacturer’s instructions. Reverse transcription and real-time PCR using Sybr Green was then applied using the same procedure as used above for SAN tissue. TGF-β1 at the transcription level was thus detected. At the same time the culture medium was collected to assess levels of activated TGF-β1 protein by TGFβ1 Emax® ImmunoAssay System (Promega, UK) following manufacturer’s instructions.

Computer simulations

This study has shown that, with ageing and on knockout of one copy of the SCN5A gene, there are many changes in ion channels at the mRNA level. To consider the possible consequences of these changes, computer modelling was used. Pacemaking cycle length of the SAN cell was simulated.

The SAN action potential (AP) of the young WT mouse was simulated using a model of the action potential of a mouse sinus node myocyte from Kharche et al. 7 To simulate the SAN AP of the old WT mouse and young and old Scn5a+/- mice, the ionic conductance for each of the major ionic currents in the Kharche et al. model 7 were modified based on changes in ion channel expression at the mRNA level. In the old WT mouse and young and old Scn5a+/- mice, the percentage change in the level of mRNA (with respect to that in the young WT mouse) for the ion channels responsible for a particular ionic current was used to adjust the ionic conductance for the ionic current in the Kharche et al. model. For example, in the young Scn5a+/- mouse, Nav1.5 mRNA was 41% of that in the young WT mouse (Table S1). We assumed, therefore, that in the young Scn5a+/- mouse the Na+ conductance governing the TTX-resistant Na+ current, I_{Na}, was also 41% of that in the young WT mouse. In cases in which more than one ion channel is responsible for an ionic current (I_{Ca,T}, I_{K1} and I_{K1}), the expression levels of the relevant ion channel mRNAs (Cav3.1 and Cav3.2 in the case of I_{Ca,T}, Kv4.2
and Kv4.3 in the case of $I_{\text{K},1}$ and Kir2.1 and Kir2.2 in the case of $I_{\text{K},1}$) were summed after correction for (multiplication by) the single channel conductance (Table S3). The single channel conductances of Cav3.1 and Cav3.2 are taken from Ono and Iijima, $^8$ that of Kv4.2 is taken from Wang et al., $^9$ that of Kv4.3 is taken from Holmqvist et al. $^{10}$ and those of Kir2.1 and Kir2.2 are taken from Liu et al. $^{11}$ In the Kharche et al. model, $^7$ Na$^+$-Ca$^{2+}$ exchange, sarcoplasmic reticulum Ca$^{2+}$ release and sarcoplasmic reticulum Ca$^{2+}$ uptake was scaled in an analogous way (depending on expression of NCX1, RyR2 and SERCA2A mRNAs, respectively; Table S3). Coupling conductance between nodal and atrial cells was calculated in an analogous way – in this case, the expression levels of mRNAs for four connexin isoforms (Cx30.2, Cx40, Cx43 and Cx45) were summed after correction for (multiplication by) the single channel conductance (Table 3S). The single channel conductance of the connexins are taken from Boyett et al. $^{12}$ The coupling conductance between the nodal and atrial cells of the young WT mouse was set to obtain a reasonable conduction time and this was then scaled (depending on the connexin mRNA expression) for the other mice.

The conduction time between the nodal and atrial cells (measured as the time delay ($\Delta t$) between the excitation of the SAN and atrial cell) were computed. As there is no murine atrial cell model available, the Kharche et al. model for mouse SAN was modified by removing the pacemaking currents ($I_{\text{f}}$ and $I_{\text{CaT}}$) to reproduce atrial action potential. The coupled sinus node and atrium system was as:

$$C_s \frac{dV_s}{dt} = -I_{\text{tot},s} + I_j$$

$$C_a \frac{dV_a}{dt} = -I_{\text{tot},a} - I_j$$

$$I_j = g(V_a - V_s)$$

where subscripts $s$ and $a$ denote SAN and atrial cell respectively. $C$ is the membrane capacitance ($\mu$ F/cm$^2$), $V$ the membrane potential (in mV), $t$ the time (in S) and $I_{\text{tot}}$ the total current (in nA). The coupling between the SAN and the atrial cell is through the junction current $I_j$; and $g$ is the junction (or coupling) conductance (in nS) specifying the coupling strength between these two cells.

The modelling above assumes that there is a linear relationship between mRNA and function and it is known that this is not necessarily the case. However, the aim of the modelling is not to produce a definitive biophysically-detailed AP model - instead it is to consider the possible consequences for the AP of the changes in expression levels of the ion channels etc. It is, therefore, a form of bioinformatics. We have previously used an analogous approach to compute the human SAN AP. $^{13}$
Supplemental References


Supplementary Tables

Table S1. Simulation of the sinus node action potential based on changes in gene expression. The table shows different processes (major ionic currents, intracellular Ca\(^{2+}\) processes), the ion channels (or exchanger or pump) responsible for the different processes (and if needed the single channel conductance of the ion channel), the expression level of the mRNA responsible for the different ion channels etc. in the sinus node of the four types of mice studied, the sum of mRNAs responsible for the different processes, and finally the sum of mRNAs expressed as a percentage of that in the sinus node of the young WT mice. In cases where more than one transcript is responsible for a process, prior to summing, the expression level was multiplied by the single channel conductance.

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<th>Process</th>
<th>Channels</th>
<th>g (pS)</th>
<th>Young WT (2-ΔCT)</th>
<th>Young Scn5a(^{+/−}) (2-ΔCT)</th>
<th>Old WT (2-ΔCT)</th>
<th>Old Scn5a(^{+/−}) (2-ΔCT)</th>
<th>Young WT (sum)</th>
<th>Young Scn5a(^{+/−}) (sum)</th>
<th>Old WT (sum)</th>
<th>Old Scn5a(^{+/−}) (sum)</th>
<th>Young WT (%)</th>
<th>Young Scn5a(^{+/−}) (%)</th>
<th>Old WT (%)</th>
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Table S2. ECG parameters from young WT, young Scn5a<sup>+/−</sup>, old WT and old Scn5a<sup>+/−</sup> anesthetised mice.

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AR: adrenergic receptor
Legends to Supplemental Figures

**Figure S1.** A, Sino-atrial preparation obtained from WT mice for electrical mapping. CT, crista terminalis; RA, right atrium; IVC, inferior vena cava; SVC, superior vena cava; SEPT, septum; V, ventricle. The area within the dashed line represents where the EPC probe places. B, multi-electrode array of 64 electrodes, giving a total dimension of 3.7×4.5 mm.

**Figure S2.** Immunolabelling of HCN4 in the SAN tissue section by anti-HCN4 antibody. Scale bar, 50 μm. Nodal artery was observed in the middle of the SAN.

**Figure S3.** Nav1.5 channel activity in cultured cardiac myocytes. A. Examples of immunolabelling of Nav1.5 channels in cells with (left panel) and without (middle panel) treatment of 15 μg/ml Nav1.5-E3 Ab for 24 hours. Scale bar: 20 μm. Channel expression intensities was quantified by Velocity in control myocytes (108 ± 7 n=11) and Nav1.5-E3 Ab treated myocytes (83 ± 8 n=10) B. Representative $I_{Na}$ recorded in non-treated (left panel) and treated myocytes (middle panel) with 24-hour incubations with Nav1.5-E3 Ab for 24 hours. Current density in control myocytes (66 ± 8 pA/pF, n=6) and Nav1.5-E3 Ab treated myocytes (66 ± 8 pA/pF, n=6).
Online Fig 2

A

Control

E-3-Nav1.5 Ab

P<0.05

B

Control

E-3-Nav1.5 Ab

P<0.01

Fig.S3