Multiple Loss-of-Function Mechanisms Contribute to \textit{SCN5A}-Related Familial Sick Sinus Syndrome

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Abstract

\textbf{Background:} To identify molecular mechanisms underlying \textit{SCN5A}-related sick sinus syndrome (SSS), a rare type of SSS, in parallel experiments we elucidated the electrophysiological properties and the cell surface localization of thirteen human \textit{Na}_{1.5} (h\textit{Na}_{1.5}) mutant channels previously linked to this disease.

\textbf{Methodology/Principal Findings:} Mutant h\textit{Na}_{1.5} channels expressed by HEK293 cells and \textit{Xenopus} oocytes were investigated by whole-cell patch clamp and two-microelectrode voltage clamp, respectively. HEK293 cell surface biotinylation experiments quantified the fraction of correctly targeted channel proteins. Our data suggested three distinct mutant channel subtypes: Group 1 mutants (L212P, P1298L, Delf1617, R1632H) gave peak current densities and cell surface targeting indistinguishable from wild-type h\textit{Na}_{1.5}. Loss-of-function of these mutants resulted from altered channel kinetics, including a negative shift of steady-state inactivation and a reduced voltage dependency of open-state inactivation. Group 2 mutants (E161K, T220I, D1275N) gave significantly reduced whole-cell currents due to impaired cell surface localization (D1275N), altered channel properties at unchanged cell surface localization (T220I), or a combination of both (E161K). Group 3 mutant channels were non-functional, due to an almost complete lack of protein at the plasma membrane (T187I, W1421X, K1578fs/52, R1623X) or a probable gating/permeation defect with normal surface localisation (R878C, G1408R).

\textbf{Conclusions/Significance:} This study indicates that multiple molecular mechanisms, including gating abnormalities, trafficking defects, or a combination of both, are responsible for \textit{SCN5A}-related familial SSS.

Introduction

Sick sinus syndrome (SSS) was first described nearly 40 years ago by Lown as a complicating arrhythmia following cardioversion [1]. Today SSS (also called sinus node dysfunction, SND) refers to abnormalities in sinus node impulse formation and propagation, including sinus bradycardia, sinus pause/arrest, chronotropic incompetence, and sinoatrial exit block [2]. SSS is frequently associated with conduction system disease in the heart and various supraventricular tachyarrhythmias, such as atrial fibrillation and atrial flutter. When associated with supraventricular tachyarrhythmias, SSS is often termed tachy-brady syndrome [2]. SSS accounts for approximately 50% of the million permanent pacemaker implants per year worldwide [3]. SSS may be associated with underlying cardiac disease conditions including any form of acquired heart disease (e.g. coronary artery disease, cardiomyopathies and valvular heart disease), and occurs following surgical injury. However, SSS most commonly occurs in the elderly in the absence of an apparent accompanying heart disease [4].

Voltage-gated Na\textsuperscript{+} channels mediate the rapid upstroke of the action potential in excitable tissues. \textit{Na}_{1.5}, encoded by the \textit{SCN5A} gene, is the predominant isoform in the heart of higher mammals [5,6]. Mutations in \textit{SCN5A} have been linked to various cardiac arrhythmic syndromes ranging from acute life-threatening tachyarrhythmias to bradyarrhythmias: the congenital long QT syndrome subtype 3 (LQT3) [7,8], Brugada syndrome (BrS) [9–11], isolated cardiac conduction disease (CCD) [12], and sudden infant death syndrome (SIDS) [13].

Recently, a number of studies have linked genetic defects in ion channels, including human \textit{Na}_{1.5} (h\textit{Na}_{1.5}), to familial SSS. To date, fourteen \textit{SCN5A} mutations have been associated with this disease [14,15]. Although \textit{SCN5A}-related familial SSS is rare, reports have conclusively demonstrated that h\textit{Na}_{1.5} plays essential roles in the excitation of atrial and ventricular myocytes, and in impulse generation and propagation by the sinus node [14,16–20]. Clinical data on \textit{SCN5A}-related SSS are strongly supported by several experimental results. Transcription and expression of \textit{Na}_{1.5} mRNA and protein respectively in the human peripheral sinus node was demonstrated recently.
by Chandler et al. [21]. Na\textsuperscript{+} currents were recorded in sinus node pacemaker cells from both mammalian animals [14,22] and humans [23]. Importantly, reduced Na\textsuperscript{+} channel expression in the hearts of heterozygous SCN5A\textsuperscript{-/-} mice resulted in sinus bradycardia, slowed sinoatrial conduction, and sinoatrial exit block; these phenomena are also observed in SSS patients [24].

Most of these SSS-related mutant channels have been expressed by different heterologous host cells including mammalian cell lines and *Xenopus* oocytes, and several loss-of-function features have been identified [22]. However, electrophysiological recordings have not yet been combined with biochemistry techniques that enable quantitative estimation of the localization of mutant channel proteins at the plasma membrane. Together, these data would enable discrimination between electrical defects of correctly targeted mutant channels and impaired subcellular localization. Furthermore, none of the mutant channels has been expressed by parallel systems in the same lab. Previous studies, for example results for BrS mutation T1620M [25], suggest that loss-of-function properties depend on the expression system chosen. Similarly, minor defects were observed for the familial SSS-related mutant channel D1275N when expressed by *Xenopus* oocytes [17]; however, it has been speculated that more severe defects might be observed upon expression by a mammalian cell line [26,27].

In this study, we have investigated the molecular mechanisms underlying loss-of-function of hNa\textsubscript{1.5} mutant channels associated with familial SSS. The first aim was to investigate both the electrophysiological properties and the plasma membrane localization of thirteen mutant channels in HEK293 cells: This would enable us to discriminate between protein targeting and electrophysiological defects. The second aim was to compare the electrophysiological properties of nine selected mutant channels expressed by both HEK293 cells and *Xenopus* oocytes: This would identify any dependence of results upon these commonly used expression systems. Our results provide novel insights into the multiplicity of molecular mechanisms underlying SCN5A-related SSS, and hence a better understanding of genotype-phenotype correlations for this rare channelopathy.

### Results

**Electrophysiological properties of mutant hNa\textsubscript{1.5} channels in HEK293 cells**

Thirteen hNa\textsubscript{1.5} mutants previously identified in familial SSS patients were expressed heterologously by HEK293 cells and characterized by whole-cell patch clamp recordings. For ease of description, these mutants were classified into three groups according to the size of the whole-cell Na\textsuperscript{+} currents (Fig. 1). Group 1 mutants (L212P, P1298L, DelF1617, and R1632H) generated peak Na\textsuperscript{+} currents comparable to those observed for wild-type hNa\textsubscript{1.5} (Table 1, Figs. 1A and 2A). Group 2 mutants (E161K, T220I, and D1275N) produced significantly reduced but detectable whole-cell Na\textsuperscript{+} currents (Table 1, Figs. 1B and 3A). Group 3 mutants (T187I, R978C, G1408R, W1421X, K1578fs/52, and R1623X) did not produce any detectable Na\textsuperscript{+} inward current (Fig. 1C).

A feature common to all group 1 mutants was a significant shift of steady-state inactivation towards hyperpolarized potentials (Table 1, Fig. 2C). This is likely to result in reduced channel availability in patients’ cardiomyocytes. The largest shift of the mid-inactivation potential was observed for R1632H (−20.7 mV). Shifts of −10.5 mV, −10.6 mV, and −8.9 mV were recorded for L212P, DelF1617, and P1298L respectively. The left-shift of steady-state inactivation was accomplished either by an unchanged steady-state activation (P1290L, R1632H) or by a left-shift of steady-state activation (−15.0 mV for L212P, −6.8 mV for DelF1617; Table 1, Fig. 2B). Left shifts of steady-state activation towards hyperpolarized potentials may partially antagonize loss-of-function caused by reduced channel availability. Recovery from inactivation by group 1 mutants was unchanged (L212P, P1290L), slightly accelerated (DelF1617), or dramatically decelerated (R1632H; Fig. 2D). Full recovery of R1632H at −120 mV took 5 seconds. This suggests that most R1632H channels are inactivated at physiological membrane potentials and normal heart rates.

When considering inactivation time constants as a function of the membrane voltage (Fig. 2E and 2F), we observed a second feature common to all group 1 mutants: The voltage dependency...
of the inactivation process was reduced, resulting in a faster current decay and hence loss-of-function at less depolarized membrane potentials. In addition, Delf1617 and R1632H displayed inactivation defects at more depolarized membrane voltages and hence a potential gain-of-function mechanism (Fig. 2F). For L212P, lower inactivation time constants at more negative potentials can be at least partially explained by the pronounced negative shift of the mid-activation potential by −15 mV (Fig. 2E).

In contrast to the clear hyperpolarizing shift of steady-state inactivation seen with group 1 mutant channels, availability of group 2 mutants was either unchanged (E161K, D1275N) or only slightly reduced (shift by −5.6 mV for T220I; Table 1, Fig. 3C). Steady-state activation curves were shifted by +19.8 mV and +3.1 mV towards depolarizing potentials for E161K and D1275N, respectively (Table 1, Fig. 3B). Positive shifts were not observed for group 1 mutants. In addition to the reduced current densities, the positive shifts of steady-state activation can be expected to further impair excitability of cardiomyocytes [28].

Electrophysiological properties of hNa1.5 mutant channels in Xenopus oocytes

In some previous reports, differences between electrophysiological properties reported for the same hNa1.5 mutant channel were attributed to the use of different expression systems [25]. Compared to Xenopus oocytes, HEK293 or CHO expression systems are often considered more reliable because of their mammalian origin and because of their cultivation at body core temperature. Xenopus oocytes injected with mutant cRNA are incubated for several days at 18°C, which may stabilize mutant channels resulting in a partial restoration of functional surface expression. In order to investigate whether similar electrophysiology results would be obtained from mutant channels expressed by

| Channel | peak current density (pA/pF) | Steady-state activation | Steady-state inactivation | Recovery from inactivation |
|---------|-----------------------------|-------------------------|---------------------------|----------------------------|
|         |                             | k (mV) | V1/2 (mV) | n | k (mV) | V1/2 (mV) | n | t<sub>r</sub> (ms) | A<sub>r</sub> | t<sub>a</sub> (ms) | A<sub>a</sub> | n |
| hNa1.5  |                             | −866.5 ± 79.8 | 6.9 ± 0.2 | | −34.7 ± 0.7 | 23 | −6.4 ± 0.1 | | −81.4 ± 0.7 | 24 | 6.9 ± 0.3 | 0.89 ± 0.01 | 149.2 ± 11.8 | 0.11 ± 0.01 | 20 |
| Group 1 mutants |
| L212P   | −805.3 ± 76.7 | 8.0 ± 0.3 | −49.7 ± 0.8 | 22 | −5.5 ± 0.1 | −91.9 ± 0.8 | 28 | 7.5 ± 0.4 | 0.86 ± 0.02 | 159.9 ± 12.1 | 0.14 ± 0.02 | 19 |
| P1298L  | −789.6 ± 84.0 | 7.2 ± 0.2 | −35.2 ± 0.6 | 19 | −6.8 ± 0.1 | −90.3 ± 0.8 | 24 | 7.4 ± 0.3 | 0.90 ± 0.01 | 194.6 ± 17.0 | 0.10 ± 0.01 | 22 |
| Delf1617| −687.8 ± 64.9 | 7.9 ± 0.2 | −41.5 ± 1.2 | 24 | −6.7 ± 0.1 | −92.0 ± 1.1 | 25 | 6.0 ± 0.4 | 0.80 ± 0.01 | 108.4 ± 8.39 | 0.19 ± 0.01 | 18 |
| R1632H  | −1105.6 ± 112.0 | 7.0 ± 0.2 | −36.1 ± 0.8 | 21 | −7.2 ± 0.1 | −102.1 ± 0.8 | 25 | 114.3 ± 11.8 | 0.28 ± 0.02 | 695.8 ± 36.4 | 0.72 ± 0.02 | 23 |
| Group 2 mutants |
| E161K   | −203.7 ± 30.8 | 9.0 ± 0.1 | −14.9 ± 0.7 | 19 | −6.6 ± 0.1 | −79.9 ± 0.9 | 21 | 6.5 ± 0.4 | 0.86 ± 0.02 | 192.3 ± 47.8 | 0.12 ± 0.02 | 14 |
| T220I   | −533.8 ± 56.6 | 7.3 ± 0.1 | −35.7 ± 0.7 | 25 | −6.5 ± 0.1 | −87.0 ± 0.8 | 30 | 8.0 ± 0.4 | 0.86 ± 0.01 | 123.2 ± 8.8 | 0.137 ± 0.01 | 21 |
| D1275N  | −575.0 ± 57.6 | 6.8 ± 0.2 | −31.6 ± 0.8 | 18 | −5.5 ± 0.1 | −79.6 ± 0.6 | 21 | 3.9 ± 0.1 | 0.89 ± 0.01 | 140.4 ± 10.4 | 0.107 ± 0.01 | 23 |

*indicates p<0.05 versus hNa1.5.
**indicates p<0.01 versus hNa1.5.
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Table 1. Electrophysiological properties of wild-type and mutant hNa1.5 channels in HEK293 cells.

For E161K, somewhat reduced (shift by −6.6 mV) for T220I and D1275N, respectively (Table 1, Fig. 3B). Positive shifts were not observed for group 1 mutants. In addition to the reduced current densities, the positive shifts of steady-state activation can be expected to further impair excitability of cardiomyocytes [28].

Steady-state activation curves were shifted by +19.8 mV and +3.1 mV towards depolarizing potentials for E161K and D1275N, respectively (Table 1, Fig. 3B). Positive shifts were not observed for group 1 mutants. In addition to the reduced current densities, the positive shifts of steady-state activation can be expected to further impair excitability of cardiomyocytes [28].

Electrophysiological properties of wild-type and mutant hNa1.5 channels in HEK293 cells.

In conclusion, during comparisons between SSS-related mutant channels tested (T187I, R176C, G1408R, W1421X), we were unable to detect any Na<sup>+</sup> current even after injecting undiluted cRNA, supporting our data from HEK293 cells. Neither the lower incubation temperature nor a high cRNA concentration could restore functional expression. For the other channel variants, classified upon expression by HEK293 cells as group 1 mutants (L212P, P1298L) or group 2 mutants (E161K, T220I, D1275N), we also observed data similar to those obtained from HEK293 cells (Fig. 4, Table 2). Only a few results differed from those obtained from transfected HEK293 cells: First, normalized peak currents obtained from oocytes were significantly smaller for E161K, D1275N and even for P1298L, which was classified as a group 1 mutant according to results from HEK293 cells (Figs. 1 and 4). For E161K, currents were clearly above background but too small to ensure a reliable evaluation of channel kinetics. Second, the mid-inactivation potential for D1275N was shifted towards hyperpolarized potentials only for oocytes, suggesting a contribution of reduced channel availability to the patients’ phenotype. Third, recovery from inactivation by L212P was accelerated in oocytes but not in HEK cells, indicating that only the oocyte expression system revealed this potential loss-of-function mechanism. Fourth, minor differences between the two expression systems were as follows: a slightly altered slope of the steady-state activation curve for D1275N channels in oocytes only; a slightly altered slope of the steady-state inactivation curve for P1298L channels in HEK293 cells only; and a slight increase of the fast recovery time constant for T220I channels in HEK293 cells (compare Tables 1 and 2). In conclusion, during comparisons between SSS-related mutant channels and wild-type hNa1.5, most of the electrophysiological parameters obtained from oocytes were not different from respective results obtained from HEK293 cells (Tables 1 and 2). Notably, for three out of the four mutant channels that showed expression system-dependent properties, more severe loss-of-function features were associated with oocytes: This suggests that results from oocytes may account better for cardiac excitation abnormalities observed in familial SSS.
Figure 2. Electrophysiological properties of four group 1 mutant channels expressed by HEK293 cells. Individual parameters are summarized in Table 1. (A) Peak current-to-voltage (I–V) relationships. Currents were elicited from the holding potential of −120 mV to the test potentials indicated. (B) Steady-state activation as a function of voltage. (C) Steady-state inactivation as a function of voltage. (D) Recovery from inactivation. Normalized data were fitted to double exponentials yielding the fast and slow time constants listed in Table 1. (E, F) Time constant of inactivation (θ) as a function of voltage. Parameters were obtained from monoexponential fits. Error bars represent the mean ± SEM. doi:10.1371/journal.pone.0010985.g002
Evaluation of the plasma membrane targeting using cell surface biotinylation experiments

We performed cell surface biotinylation experiments in conjunction with Western blotting using HEK293 cells transfected with the thirteen familial SSS-related mutants. For each mutant, the fraction of correctly targeted channel to the total cellular channel pool was determined and normalised with the corresponding fraction determined for wild-type from the same blot. Each calculation was performed in (at least) triplicate to enable the determination of statistically significant differences. Representative Western blots are shown in Fig. 5; normalized cell surface signals are shown alongside peak current densities in Fig. 6.

As expected from our whole-cell current measurements of HEK293 cells, all group 1 mutants were present at the plasma membrane in quantities similar to that for wild-type hNav1.5. For group 2 mutants, which generated smaller whole-cell currents

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**Figure 3. Electrophysiological properties of three group 2 mutant channels expressed by HEK293 cells.** Individual parameters are summarized in Table 1. (A) Peak current-to-voltage (I–V) relationships. Currents were elicited from the holding potential of −120 mV to the indicated test potentials. (B) Steady-state activation as a function of voltage. (C) Steady-state inactivation as a function of voltage. (D) Recovery from inactivation. (E) Time constant of inactivation (\(\tau_i\)) as function of voltage. Error bars represent the mean ± SEM.

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Loss-of-function mechanisms

This study evaluated cell surface localization of thirteen hNa,1.5 mutant channels previously linked to familial SSS, and correlated these data with the electrophysiological properties of the mutant channels. Whereas several electrophysiological parameters for some of the mutant channels have been reported by other groups, little or no information was available on the contribution of possible maturation/trafficking defects to losses-of-function for the mutants. Hence, this study provides new mechanistic insight into the pathogenesis of SCN5A-related familial SSS.

Discussion

This study evaluated cell surface localization of thirteen hNa,1.5 mutant channels previously linked to familial SSS, and correlated these data with the electrophysiological properties of the mutant channels. Whereas several electrophysiological parameters for some of the mutant channels have been reported by other groups, little or no information was available on the contribution of possible maturation/trafficking defects to losses-of-function for the mutants. Hence, this study provides new mechanistic insight into the pathogenesis of SCN5A-related familial SSS.

Table 2. Electrophysiological properties of wild-type and mutant hNa,1.5 channels in Xenopus oocytes.

| Channel | Normalized peak current | Steady-state activation | Steady-state inactivation | Recovery from inactivation |
|---------|-------------------------|-------------------------|--------------------------|---------------------------|
|         | n                       | k (mV)                  | V1/2 (mV)                | n                         | tA (ms) | tA (ms) | A (s) | n |
| hNa,1.5 | 1.00±0.04               | 75                      | 3.6±0.1                  | 34.2±0.6                  | 22      | -5.5±0.1 | -67.7±0.5 | 31 | 4.8±0.3 | 1.16±0.02 | 159.3±22.2 | 0.09±0.01 | 24 |
| L212P   | 1.01±0.08               | 56                      | 4.3±0.1*                 | 46.5±0.5*                 | 22      | -4.8±0.1* | -821±0.3* | 25 | 5.7±0.2* | 1.14±0.01 | 319.0±44.3 | 0.09±0.01 | 24 |
| T220I   | 0.75±0.04*              | 62                      | 3.7±0.1                  | 35.1±0.4                  | 24      | -5.6±0.1 | -733±0.5* | 28 | 5.0±0.4 | 1.17±0.02 | 115.4±17.2 | 0.07±0.01 | 15 |
| D1275N  | 0.28±0.03*              | 37                      | 4.1±0.1*                 | 32.2±0.4*                 | 19      | -4.9±0.1* | -712±0.6* | 15 | 3.8±0.3* | 1.19±0.02 | 187.7±25.9 | 0.09±0.01 | 14 |
| P1298L  | 0.59±0.03*              | 47                      | 3.8±0.1                  | 34.3±0.3                  | 21      | -5.4±0.1 | -769±0.5* | 26 | 5.4±0.2 | 1.16±0.01 | 229.9±30.8 | 0.09±0.01 | 21 |

*indicates p<0.05 versus hNa,1.5.
**indicates p<0.01 versus hNa,1.5.

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Consequently, these mutants are largely trafficking-deficient.
In a previous study on T187I, functional expression could neither be restored by mexiletine treatment nor by co-expression of the β1 subunit [31]. A screen for known protein motifs in the affected S2–S3 linker in domain I was unsuccessful. We noticed that the distal half of this linker that included threonine 187 is highly conserved among voltage-gated Na⁺ channels. It is possible that this linker region, which is located intracellularly, contains an unknown ER export signal. Fourth, loss-of-function can also be attributed to both a partial trafficking defect and alterations of channel kinetics, as observed for E161K and D1275N (Tables 1 and 2). For E161K, the reduction of the cell surface biotinylation signal was much less pronounced than the reduction of the whole-cell current (Fig. 5), suggesting impaired single channel properties even for correctly targeted mutant channels.

**Figure 5.** Representative Western blots showing Na⁺,1.5 polypeptides purified from the surface of HEK293 cells by the biotinylation/precipitation procedure (cell surface) and the Na⁺,1.5 polypeptides in HEK293 cell lysates (total). Calnexin was used as a control to demonstrate the absence of endoplasmic reticulum membranes in the isolated plasma membrane fraction. doi:10.1371/journal.pone.0010985.g005

**Association of loss-of-function of hNa⁺,1.5 mutants to familial SSS and to other cardiac excitation disorders**

Classifications of hNa⁺,1.5 mutant channels according to current densities or trafficking properties are less helpful when explaining
mutant, produced from the other allele, can be considered as either severely affected (R1632H) or non-functional (G1408R, R1623X) [16]. This indicates that sinus node function is affected at an early developmental stage when the total Na\(^{+}\) current is reduced to less than 50% of its maximal value. The parents of the patients, carrying only one of the six mutations and hence producing a total of at least 50% of the maximum Na\(^{+}\) current from the wild-type plus the affected allele, were either asymptomatic or presented with a mild cardiac rhythm disorder (first degree AV block) [16]. Wild-type hNa\(_{1.5}\) channel activity plus the residual Na\(^{+}\) channel activity of some of the mutants were sufficient to maintain normal cardiac function. However, it must be mentioned that more severe phenotypes were observed in unrelated families carrying the heterozygous mutations G1408R, T220I, or DelF1617 (Table 3) [29,32,33]. Moreover, the heterozygous mutation R1623X caused a combination of BrS and SSS in another patient [31]. In contrast to carriers of compound heterozygous SCN5A mutations, the R1623X carrier was already 65 years old when he experienced recurrent syncpe due to sinus arrest. Similarly, several carriers of other mutations (T187I, R878C, W1421X, K1578fs, and E161K) investigated in this study also presented with other cardiac rhythm disorders, such as BrS, CCD, or AV block. Most of these patients were adults when they developed signs of SSS (Table 3). Consequently, a strong Na\(^{+}\) current reduction, arising from SCN5A haploinsufficiency, can result in sinus node dysfunction, but the disease phenotype is accompanied by other cardiac excitations disorders, and onset of symptoms occurs at a later ontogenetic stage. Notably, dozens of other severe loss-of-function mutations in SCN5A are known to cause BrS, CCD, or both [28]. However, only a few mutations have been related to sinus node dysfunction, suggesting that as yet unknown factors other than Na\(^{+}\) current reduction and aging contribute to the development of SSS in carriers of T187I, R878C, W1421X, K1578fs, and E161K.

The importance of other risk factors in SCN5A-related SSS becomes obvious when considering the striking genotype-phenotype disassociation for L212P and D1275N carriers. Atrial standstill exhibited by the L212P carrier, a three years old boy, occurred only in combination with a connexin 40 (Cx40) polymorphism [34]. The father of the index patient, an L212P carrier lacking this Cx40 variant, exhibited normal sinus rhythm [34]. The normal cardiac phenotype of this L212P carrier is in good agreement with the normal trafficking and the mildly affected electrophysiological properties of L212P carriers (Table 3). Similar observations were reported for D1275N [17]: The study by Groenewegen et al. suggested a rare Cx40 polymorphism as a prerequisite for atrial standstill in D1275N carriers [17]. However, D1275N was correlated in unrelated families to sinus node dysfunction, arrhythmia, and ventricular dilation [26], to atrial fibrillation, SSS, CCD, and DCM [32], and to atrial arrhythmia and CCD [27]. The reasons for this spectrum of disease manifestations are unknown, and it is hard to imagine that the minor changes of channel kinetics associated with D1275N are responsible for this variety. Recently, we found that the common SCN5A polymorphism H558R enhanced trafficking of D1275N [35]. Consequently, it is reasonable to assume that several factors, such as polymorphisms in other cardiac genes, gender differences, modulatory proteins of hNa\(_{1.5}\) [36], and/or ageing and fibrosis, are important determinants in SCN5A-related channelopathies, including familial SSS.

**Limitations of this study**

This study possesses a number of limitations. First, caution should be taken when extrapolating the findings from this *in vitro*...
study of HEK293 cells and oocytes to the more complex in vivo conditions. A bridge to the human in vivo situation might be provided by genetically modified mouse models that could possibly provide further information. Second, this study was unable to examine other contributing factors, such as the fibrosis, the remodeling of ion channels, and the autonomic dysfunction that occur as a result of ageing. Third, the effects of different β subunits upon the biophysical properties and intracellular trafficking of the Na⁺ channel α subunit have not been explored in this study. Accessory β subunits are well-known modulators of voltage-gated Na⁺ channels, and it is possible that the different β subunits exert distinct effects on the SSS-associated mutant channels investigated in this study.

Materials and Methods

Recombinant DNA procedures

Plasmid pSP64T-hH1 encoding hNa,1.5 (also called hH1, accession No. M77235) was kindly provided by Dr. A. L. George (Vanderbilt University, TN, USA). For expression in HEK293 cells, the hNa,1.5 cDNA was subcloned into the pIRES2-EGFP expression plasmid (Clontech, CA, USA) using SalI/SmaI restriction sites. All mutations were then introduced by site-directed mutagenesis (QuickChange® II XL Site-Directed Mutagenesis Kits, Stratagene, CA, USA) according to the manufacturer’s instructions. DNA constructs were checked by restriction digests and DNA sequencing.

Transfection and electrophysiological recordings using HEK293 cells

A human embryonic kidney cell line (HEK293 cell line, CRL-1573, supplied by American Type Culture Collection, USA) was cultured in Dulbecco’s Modification of Eagle’s Medium (DMEM, Lonza, Belgium) supplemented with 10% fetal bovine serum (Lonza, Belgium), 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were transfected using a standard calcium phosphate precipitation method using 0.2 μg DNA per 35 mm dish for electrophysiological experiments, or Lipofectamine (Invitrogen) using 1.5 μg DNA per 60 mm dish for cell surface biotinylation experiments.

For electrophysiological recordings, HEK293 cells were trypsinized 24 h after transfection and seeded onto a glass coverslip at a density that enabled single cells to be identified. Whole-cell Na⁺ currents were recorded using the patch-clamp technique, a 200B amplifier (Axon Instruments, Foster City, CA, USA), and with patch pipettes fabricated from borosilicate glass capillaries (1.5 mm outer diameter; Fisher Scientific, Pittsburgh, PA, USA). The pipettes were pulled with a PP-830 gravity puller (Narishige, Tokyo, Japan), and filled with a pipette solution of the following composition (in mmol/L): CsCl 130, NaCl 10, HEPES 10, EGTA 10, pH 7.2 (CsOH). Pipette resistance ranged from 1.0 to 2.0 MΩ when the pipettes were filled with the internal solution. The perfusion solution contained (in mmol/L): NaCl 140, KCl 4, CaCl₂ 1.8, MgCl₂ 1.0, HEPES 10, and glucose 10, pH 7.4 (NaOH). Series resistance errors were reduced by approximately...
Table 3. Correlation between SCN5A mutations, the patients' phenotypes, and electrophysiological properties of hNav1.5 mutant channels associated with SSS and other SCN5A channelopathies.

| SCN5A genotype | SSS phenotype | Age of onset of symptoms (years) | Other reported phenotypes | Surface expression | Peak current density | Steady-state availability | Steady-state activation | Current decay | Recovery | References |
|----------------|---------------|---------------------------------|---------------------------|-------------------|---------------------|------------------------|------------------------|--------------|----------|------------|
| P1298L         | 1/3/n.s.      | 2–7                             | none reported             | =                 | ↓ a)                | reduced                | faster                 | =            |          | [16,29]   |
| G1408R         | BrS, CCD      | =                               | no current                |                   |                     |                        |                        |              |          |            |
| DelF1617       | 1/1/n.s.      | 5                               | BrS, LQT3                 | =                 | =                   | reduced                | negative shift        | faster         | faster   | [16,33,41]|
| R1632H         | AV block      | =                               | =                         | reduced           | =                   | faster                 | faster                 | slower        |          |            |
| T220I          | 1/1/n.s.      | 9                               | DCM                       | =                 | ↓                   | reduced                | faster                 | slower        |          | [16,31,32]|
| R1623X         | BrS, AV block | ↓                               | no current                |                   |                     |                        |                        |              |          |            |
| T187I          | 1/2/positive  | 33                              | BrS                       | ↓                 | no current          |                        |                        |              |          | [31]      |
| R878C          | 1/2/positive  | 8–20                            | ST elevation              | =                 | no current          |                        |                        |              |          | [30]      |
| W1421X         | 1/2/n.s.      | 2–30                            | CCD, AV block             | ↓                 | no current          |                        |                        |              |          | [42]      |
| K1578fs/S2     | 1/1/n.s.      | 68                              | BrS, AV block             | ↓                 | no current          |                        |                        |              |          | [31]      |
| E161K          | 2/6/positive  | 47                              | BrS, CCD                  | ↓                 | ↓                   | =                      | positive shift        | slower        | =         | [20]      |
| L21P           | 1/1/positive  | 3                               | none reported             | =                 | =                   | reduced                | negative shift        | faster         | slower   | [34]      |
| D1275N         | 2/17/positive | 12–34                           | DCM, CCD, AF, AV block    | ↓                 | ↓                   | reduced b)             | positive shift        | =            | faster   | [26,32]   |

Loss-of-function features in heterologously expressed hNa1.5 mutant channels, compared to wild-type hNa1.5, are highlighted (bold). F - number of affected families, P - number of patients carrying the mutation(s), I - inheritability for SSS, n.s. - not shown.

a)Observed only in Xenopus oocytes.
b)Observed only in HEK293 cells.

Reduced voltage dependency of channel inactivation, resulting in faster current decay at less depolarized potentials and slower decay at more depolarized potentials (Fig. 2F).

c) Reduced voltage dependency of channel inactivation, resulting in faster current decay at less depolarized potentials and slower decay at more depolarized potentials (Fig. 2F).

d)R1623X alone caused BrS plus SSS in another unrelated patient [31].

e)Atrial standstill in this patient was related to a Cx40 polymorphism [31].

f)Observed only in Xenopus oocytes.

References:
[16,29,30,31,32,33,34,41,42]
70–80% with electronic compensation. Signals were acquired at 50 kHz (Digitata 1440A; Axon Instruments) and analyzed with a PC running PCLAMP 10 software (Axon Instruments). All recordings were made at room temperature (20–22°C).

Expression in Xenopus laevis oocytes

For in vitro transcription, the coding regions of all constructs were cloned into the pSP64Poly(A) vector (Promega, WI, USA). The preparation of oocytes from Xenopus laevis, in vitro transcription and cRNA injection were performed according to established procedures [37]. For current comparisons, the cRNA concentration for each variant was adjusted to ~0.04 μg/μL (40 and 60 nL per oocyte) by dilution. After 3 days of incubation at 18°C in Barth medium, the whole-cell hNaL1.5 peak current amplitude was usually between 0.5 and 6.0 μA. Measurements were repeated for at least 5 different batches of oocytes. Whole-oocyte Na+ currents were recorded with the two-microelectrode voltage clamp technique using an OC725C amplifier (Warner Instruments, Weimar, Fachgebiet Tierschutz, no. 740-2684-04-02-73/96), and approved by the local government office (Thüringer Landesamt für Umwelt, Fachgebiet Tierschutz, no. 740-2684-04-02-73/96), and conformed to both institutional and NIH guidelines [38].

Pulse protocols and evaluation of electrophysiological data from HEK293 cells and oocytes

steady-state activation was determined by applying test potentials from −120 to 50 mV in 5 mV or 10 mV increments at a pulsing frequency of 0.5 Hz. Data were fitted to the Boltzmann equation $G_{Na} = \frac{1 + \exp(V_{1/2} - V/k)}{1 + \exp(-V/V_{rev})}$ where $V_{1/2}$ is the half-maximal voltage, $k$ is the slope factor, and the $Na^+$ conductance $G_{Na}$ is defined by $G_{Na} = I_{Na,peak}/(V - V_{rev})$. Here, $I_{Na,peak}$ is the normalized peak current, and $V_{rev}$ is the reversal potential.

steady-state inactivation was determined with a double pulse protocol consisting of 500 ms pre-pulses from the holding potential of −120 mV (or −140 mV for R1632H only) to voltages between −140 and −20 mV (or −160 and −20 mV for R1632H only), followed by a constant test pulse of 200 ms duration to −20 mV at a pulsing frequency of 0.5 Hz. The amplitude of peak current $I_{Na}$ during the test pulse was normalized to the maximum peak current $I_{Na,max}$ and plotted against the prepulse potential. Data were fitted with the Boltzmann function $I_{Na} = I_{Na,max}\cdot\left[1 + \exp\left(-\frac{V_{1/2} - V}{k}\right)\right]^{-1}$, where $V$ is the test potential, and $V_{1/2}$ and $k$ are as defined above.

Recovery from inactivation was determined with a double pulse protocol consisting of 500 ms pre-pulses to −20 mV, followed by variable recovery intervals at −120 mV, and a constant 20 ms test pulse to −20 mV at a pulsing frequency of 0.25 Hz (or 0.15 Hz for R1632H only). The normalized peak current amplitude ($I_{Na,peak} = I_{Na}/I_{Na,max}$) elicited by the test pulse was plotted against the recovery interval. Data were fitted with 2 exponentials: $I_{Na,peak} = A_{r} [1 - \exp(-t/\tau_{f})] + A_{s} [1 - \exp(-t/\tau_{s})]$, where $t$ is the recovery time interval, $\tau_{f}$ and $\tau_{s}$ represent fast and slow time constants respectively, and $A_{r}$ and $A_{s}$ represent fractional amplitudes of fast and slow recovery components, respectively.

Cell surface biotinylation and Western blotting

Cell surface biotinylation was performed as described previously [39]. Briefly, cell membrane proteins were labelled by incubation (4°C, 30 min) with 1 mg/mL EZ-Link™ sulfo-NHS-LC-Biotin (Pierce, IL, USA). Cells were lysed by RIPA buffer (0.5% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 50 mM/ol/L Tris, 150 mM/ol/L NaCl, and 1 mM/ol/L EDTA) in the presence of cocktail protease inhibitors (Roche, IN, USA). Labeled cell membrane proteins were precipitated by incubating (4°C, overnight) cell lysate (200 μL) with streptavidin-agarose beads (150 μL, Amersham NJ, USA). The beads were recovered by centrifugation (15000g, 15 min, 4°C), and the supernatant was retained. The beads were washed with RIPA buffer. Biotinylated cell membrane proteins were eluted from the beads into Laemmli buffer by rotation. Both the cell lysate (0.6%) and the purified biotinylated cell surface proteins (50%) were analysed by SDS-PAGE (6% acrylamide) followed by Western blotting as previously described [39,40]. The anti-Nav1.5 antibodies (Alomone, Jerusalem, Israel) ASC-013 and ASC-005 detected full-length and truncated channels respectively. Antibody against the endoplasmic reticulum protein calnexin was used in simultaneous control experiments to verify that no cytosolic protein had been biotinylated during the procedure. Results are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed using the one-way ANOVA test with significance being assumed for $p<0.05$.

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Author Contributions

Conceived and designed the experiments: JG TW TZ ML. Performed the experiments: JG TW TZ. Analyzed the data: JG TW RPOJ TZ. Contributed reagents/materials/analysis tools: DT ML. Wrote the paper: JG TZ ML. Contributed intellectually to data interpretation and manuscript preparation: TW RPOJ DT TZ. Approved the final version of the manuscript: TW RPOJ DT TZ.

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