Variable Na$_{1.5}$ Protein Expression from the Wild-Type Allele Correlates with the Penetrance of Cardiac Conduction Disease in the Scn5a$^{+/-}$ Mouse Model


DOI 10.1371/journal.pone.0009298

Publication date 2010

Document Version Final published version

Published in PLoS ONE

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: https://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Variable Na\textsubscript{v}1.5 Protein Expression from the Wild-Type Allele Correlates with the Penetration of Cardiac Conduction Disease in the Scn5a\textsuperscript{+/−} Mouse Model

Anne-Laure Leoni\textsuperscript{1,3}, Bruno Gavillet\textsuperscript{10}, Jean-Sébastien Rougeri\textsuperscript{2}, Céline Marionneau\textsuperscript{1,3}, Vincent Probst\textsuperscript{1,2,3,4}, Solena Le Scouarnc\textsuperscript{1,3}, Jean-Jacques Schott\textsuperscript{1,2,3,4}, Sophie Demolombe\textsuperscript{1,2,3}, Patrick Bruneval\textsuperscript{6}, Christopher L. H. Huang\textsuperscript{9}, William H. Colledge\textsuperscript{8}, Andrew A. Grace\textsuperscript{8}, Hervé Le Marec\textsuperscript{1,2,3,4}, Arthur A. Wilde\textsuperscript{7}, Peter J. Mohler\textsuperscript{9}, Denis Escande\textsuperscript{1,3,4}, Hugues Abriel\textsuperscript{5}, Flavien Charpentier\textsuperscript{1,2,3,4}

1 INSERM, UMR915, l’Institut du Thorax, Nantes, France, 2 CNRS, ERL3147, Nantes, France, 3 Université de Nantes, Nantes, France, 4 CHU Nantes, l’Institut du Thorax, Nantes, France, 5 Department of Clinical Research, University of Bern, Bern, Switzerland, 6 INSERM, U652, Université Paris V, Paris, France, 7 Department of Cardiology, Academic Medical Center, Amsterdam, The Netherlands, 8 The Section of Cardiovascular Biology, Departments of Biochemistry and Physiology, University of Cambridge, Cambridge, United Kingdom, 9 Department of Internal Medicine, University of Iowa Carver College of Medicine, Iowa City, Iowa, United States of America, 10 Department of Pharmacology and Toxicology, University of Lausanne, Lausanne, Switzerland

Abstract

**Background:** Loss-of-function mutations in SCN5A, the gene encoding Na\textsubscript{v}1.5 Na\textsuperscript{+} channel, are associated with inherited cardiac conduction defects and Brugada syndrome, which both exhibit variable phenotypic penetrance of conduction defects. We investigated the mechanisms of this heterogeneity in a mouse model with heterozygous targeted disruption of Scn5a (Scn5a\textsuperscript{+/−} mice) and compared our results to those obtained in patients with loss-of-function mutations in SCN5A.

**Methodology/Principal Findings:** Based on ECG, 10-week-old Scn5a\textsuperscript{+/−} mice were divided into 2 subgroups, one displaying severe ventricular conduction defects (QRS interval>18 ms) and one a mild phenotype (QRS\textless;18 ms; QRS in wild-type littersmates: 10–18 ms). Phenotypic difference persisted with aging. At 10 weeks, the Na\textsuperscript{+} channel blocker ajmaline prolonged QRS interval similarly in both groups of Scn5a\textsuperscript{+/−} mice. In contrast, in old mice (>53 weeks), ajmaline effect was larger in the severely affected subgroup. These data matched the clinical observations on patients with SCN5A loss-of-function mutations with either severe or mild conduction defects. Ventricular tachycardia developed in 5/10 old severely affected Scn5a\textsuperscript{+/−} mice but not in mildly affected ones. Correspondingly, symptomatic SCN5A–mutated Brugada patients had more severe conduction defects than asymptomatic patients. Old severely affected Scn5a\textsuperscript{+/−} mice but not mildly affected ones showed extensive cardiac fibrosis. Mildly affected Scn5a\textsuperscript{+/−} mice had similar Na\textsuperscript{+} mRNA but higher Na\textsubscript{v}1.5 protein expression, and moderately larger Ih\textsubscript{q} current than severely affected Scn5a\textsuperscript{+/−} mice. As a consequence, action potential upstroke velocity was more decreased in severely affected Scn5a\textsuperscript{+/−} mice than in mildly affected ones.

**Conclusions:** Scn5a\textsuperscript{+/−} mice show similar phenotypic heterogeneity as SCN5A-mutated patients. In Scn5a\textsuperscript{+/−} mice, phenotype severity correlates with wild-type Na\textsubscript{v}1.5 protein expression.

Introduction

The cardiac voltage-gated Na\textsuperscript{+} channel Na\textsubscript{v}1.5, encoded by SCN5A gene, determines the upstroke velocity of the action potential and, in conjunction with gap junction channels and the organization of the collagenous skeleton, controls the propagation of the cardiac electrical impulse. Loss-of-function mutations of SCN5A are associated to the inherited form of progressive cardiac conduction defect [1,2] (PCCD) and to the Brugada syndrome (BrS) [3–5]. In patients with inherited PCCD, the conduction of the cardiac impulse is abnormally slow and becomes progressively slower with aging leading ultimately to atrioventricular block and pacemaker implantation in the elderly [2]. The presence of conduction defects is also one striking feature of BrS SCN5A-mutation carriers [6,7]. As for most inherited cardiac diseases, penetrance of the defect caused by SCN5A haploinsufficiency is variable among carriers of the same mutation, particularly to atrioventricular block and pacemaker implantation in the elderly [2]. The presence of conduction defects is also one striking feature of BrS SCN5A-mutation carriers [6,7]. As for most inherited cardiac diseases, penetrance of the defect caused by SCN5A haploinsufficiency is variable among carriers of the same mutation, with patients showing severe conduction alterations and others exhibiting an almost normal phenotype [2]. This demonstrates that...
factors independent of the morbid gene exert considerable influence on outcome of the inherited “channelopathy”. One obvious possibility in humans is the contribution of “modifier genes”, which could control expressivity of the monogenic defect [9].

A mouse model with targeted disruption of Scn5a has been established [9]. In previous works [10,11], we showed that heterozygous Scn5a deficient (Scn5a+/−) mice convincingly recapitulate PCCD phenotype including progressive impairment of atrial and ventricular conduction with aging. Because progressive decline in conduction velocity is also associated with myocardial rearrangements and fibrosis, we provided the first demonstration that an isolated ion channel defect can lead to myocardial structural anomalies [10]. In the present study, we demonstrate that: (i) the expression of the conduction anomalies is variable among inbred Scn5a+/− mice; (ii) Scn5a+/− mice with severe conduction defects (severe phenotype) exhibit more myocardial rearrangements with aging than mice with mild conduction defects (mild phenotype); (iii) old mice with a severe, but not with a mild phenotype, have a markedly reduced conduction reserve and show spontaneous ventricular arrhythmias; (iv) similarly, symptomatic BrS patients carrying SCN5A mutations have more pronounced conduction slowing than asymptomatic patients; and, (v) in mice, the expression of the phenotype correlates with the ability of the normal allele to control expressivity of the monogenic defect [8]. The possibility in humans is the contribution of “modifier genes”, which on outcome of the inherited “channelopathy”. One obvious factor independent of the morbid gene exert considerable influence on outcome of the inherited “channelopathy”. One obvious possibility in humans is the contribution of “modifier genes”, which could control expressivity of the monogenic defect [9].

A mouse model with targeted disruption of Scn5a has been established [9]. In previous works [10,11], we showed that heterozygous Scn5a deficient (Scn5a+/−) mice convincingly recapitulate PCCD phenotype including progressive impairment of atrial and ventricular conduction with aging. Because progressive decline in conduction velocity is also associated with myocardial rearrangements and fibrosis, we provided the first demonstration that an isolated ion channel defect can lead to myocardial structural anomalies [10]. In the present study, we demonstrate that: (i) the expression of the conduction anomalies is variable among inbred Scn5a+/− mice; (ii) Scn5a+/− mice with severe conduction defects (severe phenotype) exhibit more myocardial rearrangements with aging than mice with mild conduction defects (mild phenotype); (iii) old mice with a severe, but not with a mild phenotype, have a markedly reduced conduction reserve and show spontaneous ventricular arrhythmias; (iv) similarly, symptomatic BrS patients carrying SCN5A mutations have more pronounced conduction slowing than asymptomatic patients; and, (v) in mice, the expression of the phenotype correlates with the ability of the normal allele to control expressivity of the monogenic defect [8].

Results

Heterogeneity of the Intraventricular Conduction Defect in Scn5a+/− Mice

Young adult (10–12 week-old) Scn5a+/− mice were investigated for their ventricular conduction parameters. Figure 1A shows representative ECG recordings from a WT and two Scn5a+/− mice. The two Scn5a+/− mice clearly differed since one exhibited a markedly prolonged QRS complex whereas the other had a relatively normal one. Figure 1B displays the QRS interval distribution in 84 WT littermates and 136 Scn5a+/− young adult mice. Statistical analysis showed that the QRS interval duration was not normally distributed in the Scn5a+/− population but distributed according to two subgroups, (i) one with a severe conduction phenotype (QRS>18 ms; mean QRS = 22±0 ms; range 19–26 ms; n = 63), and (ii) the other with a mild conduction phenotype (QRS≤18 ms; mean QRS = 15±0 ms; range 12–18 ms; n = 73; p<0.05 versus WT and severe phenotype). In the WT littermate population, QRS duration ranged from 10 to 18 ms (mean QRS = 13±0 ms). Each group (WT, mild and severe) differed significantly from the two others with p values <0.001. Phenotype severity in Scn5a+/− mice was not influenced by gender (male/female ratio: 43/30 and 31/32 in mice with mild and severe conduction defects, respectively; NS). Moreover, among the 25 litters in which all Scn5a+/− mice have been phenotyped, the average proportion of mice with mild conduction defects was 0.53±0.05 (NS versus theoretical proportion of 0.5). In 2 litters, all Scn5a+/− mice had mild conduction defects (2 and 3 mice, respectively). In 2 others, all Scn5a+/− mice had, in contrast, severe conduction defects (3 mice in each). Thus, based on the QRS duration, we distinguished 3 groups of mice: WT mice, Scn5a+/− mice with a mild phenotype, i.e. with a small QRS prolongation, and Scn5a+/− mice with a severe phenotype, i.e. with a marked QRS prolongation.

Subpopulations of these 3 groups were included in a longitudinal study conducted up to the age of 80 weeks. There was a progressive increase in QRS interval with age in WT and Scn5a+/− mice, although this increase was slightly more pronounced in Scn5a+/− mice, thus confirming previous results.
and humans with markedly prolonged QRS interval.

In contrast, no statistical difference was seen in the younger versus older patients. As shown in Figure 2B, the ajmaline-induced QRS prolongation in animals with a mild phenotype was more pronounced in those with SCN5A mutations (baseline QRS prolongation in aged patients was similar in the two subgroups of Scn5a+/− mice). To estimate their conduction reserve, we challenged young (10 weeks) and old (>53 weeks) Scn5a+/− mice with the Na+ channel blocker ajmaline (20 mg/kg i.p.). As shown in Figure 2, in young mice, the effects of ajmaline on the QRS interval were modest and similar in the two subgroups of Scn5a+/− mice (+10.4±2.0 ms versus +10.4±1.2 ms in mice with mild and severe phenotype, respectively). In old mice, ajmaline prolonged more the QRS interval in animals with a severe phenotype (+12.0±0.8 ms) than in animals with a mild phenotype (+9.6±0.7 ms; p<0.05).

These mouse data were similar to the results of ajmaline tests performed in patients from Nantes cohort diagnosed with either PCCD or BrS and carrying mutations on SCN5A gene. These patients were separated in two groups based on the duration of QRS interval under baseline conditions: one with mild conduction defects (baseline QRS=108 ms) and one with severe conduction defects (QRS>108 ms; see methods section for the cutoff value definition). As shown in Figure 2B, the ajmaline-induced QRS prolongation in aged patients was more pronounced in those with severe conduction defects under baseline conditions (+55±7 ms) than in those with mild conduction defects (+34±5 ms; p<0.05). In contrast, no statistical difference was seen in the younger patients.

Thus, the conduction reserve is reduced with aging both in mice and humans with markedly prolonged QRS interval.

Table 1. ECG characteristics of wild-type (WT) and Scn5a+/− mice with mild and severe phenotype.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Age (weeks)</th>
<th>RR (ms)</th>
<th>P (ms)</th>
<th>PR (ms)</th>
<th>QRS (ms)</th>
<th>QT (ms)</th>
<th>QTc</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>16</td>
<td>11±0</td>
<td>128±3</td>
<td>15±0</td>
<td>35±1</td>
<td>14±1</td>
<td>64±1</td>
<td>58±1</td>
</tr>
<tr>
<td>14</td>
<td>31±0</td>
<td>122±3</td>
<td>16±1</td>
<td>34±1</td>
<td>16±1</td>
<td>60±2</td>
<td>54±1</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>50±1</td>
<td>122±3</td>
<td>16±1</td>
<td>35±1</td>
<td>16±1</td>
<td>58±1</td>
<td>53±1</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>81±3</td>
<td>115±11</td>
<td>15±1</td>
<td>34±1</td>
<td>16±1</td>
<td>58±1</td>
<td>54±1</td>
<td></td>
</tr>
<tr>
<td>Mild Scn5a+/−</td>
<td>20</td>
<td>12±1</td>
<td>135±3</td>
<td>18±1**</td>
<td>39±1**</td>
<td>15±0</td>
<td>67±1</td>
<td>58±1</td>
</tr>
<tr>
<td>16</td>
<td>31±0</td>
<td>127±2</td>
<td>18±1</td>
<td>39±1**</td>
<td>18±0.4**</td>
<td>61±2**</td>
<td>54±1**</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>49±1</td>
<td>130±3</td>
<td>18±0.4*</td>
<td>40±1**</td>
<td>18±0.4**</td>
<td>61±1**</td>
<td>53±1**</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>78±5</td>
<td>132±2**</td>
<td>19±1**</td>
<td>42±2**</td>
<td>20±1**</td>
<td>66±2**</td>
<td>57±2</td>
<td></td>
</tr>
<tr>
<td>Severe Scn5a+/−</td>
<td>17</td>
<td>11±1</td>
<td>142±4***</td>
<td>18±0*</td>
<td>39±1**</td>
<td>22±0.4*</td>
<td>69±1*</td>
<td>59±1</td>
</tr>
<tr>
<td>17</td>
<td>32±0</td>
<td>128±2**</td>
<td>19±1*</td>
<td>38±1***</td>
<td>23±0.0*</td>
<td>63±1**</td>
<td>55±1</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>50±2</td>
<td>124±2**</td>
<td>18±0.4**</td>
<td>39±1**</td>
<td>24±0.4**</td>
<td>62±1**</td>
<td>56±1</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>80±3</td>
<td>128±2*</td>
<td>19±1**</td>
<td>43±1**</td>
<td>25±1.4**</td>
<td>68±2**</td>
<td>59±1*</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: n, number of animals per group; RR, RR interval duration; P, P wave duration; PR, PR interval duration; QRS, QRS complex duration; QT, QT interval duration; QTc, corrected QT interval duration.

Data are mean ± sem. *, **, ***, p<0.05, p<0.01 and p<0.001 respectively versus WT (matching age); †, ††, †††, p<0.05, p<0.01 and p<0.001 respectively versus youngest age in each group.

doi:10.1371/journal.pone.0009298.t001

Reduced Conduction Reserve in Scn5a+/− Mice and Patients with SCN5A Mutations with a Severe Phenotype

To estimate their conduction reserve, we challenged young (10 weeks) and old (>53 weeks) Scn5a+/− mice with the Na+ channel blocker ajmaline (20 mg/kg i.p.). As shown in Figure 2, in young mice, the effects of ajmaline on the QRS interval were modest and similar in the two subgroups of Scn5a+/− mice (+10.4±2.0 ms versus +10.4±1.2 ms in mice with mild and severe phenotype, respectively). In old mice, ajmaline prolonged more the QRS interval in animals with a severe phenotype (+12.0±0.8 ms) than in animals with a mild phenotype (+9.6±0.7 ms; p<0.05). These mouse data were similar to the results of ajmaline tests performed in patients from Nantes cohort diagnosed with either PCCD or BrS and carrying mutations on SCN5A gene. These patients were separated in two groups based on the duration of QRS interval under baseline conditions: one with mild conduction defects (baseline QRS=108 ms) and one with severe conduction defects (QRS>108 ms; see methods section for the cutoff value definition). As shown in Figure 2B, the ajmaline-induced QRS prolongation in aged patients was more pronounced in those with severe conduction defects under baseline conditions (+55±7 ms) than in those with mild conduction defects (+34±5 ms; p<0.05). In contrast, no statistical difference was seen in the younger patients.

Thus, the conduction reserve is reduced with aging both in mice and humans with markedly prolonged QRS interval.

Extensive Cardiac Fibrosis in the Ventricular Myocardium of Scn5a+/− Mice with a Severe Phenotype

Previously, we showed that heterozygous Scn5a invalidation in mouse produces ventricular rearrangements and fibrosis with aging [10,11]. We also showed that two transcription factors, Atf5, a stress-inducible gene, and Egr1, an early growth response gene, were up-regulated with aging in Scn5a+/− mice. Ventricular sections were evaluated for the presence of fibrosis in 83±3 week-old mice. The investigator was blinded to genotype and phenotype. Scn5a+/− mice with a severe phenotype were characterized by extensive fibrosis located in the left ventricular free wall and intraventricular septum (Figure 3A). In contrast, fibrosis in Scn5a+/− mice with a mild phenotype when present was restricted to small areas. Semi-quantitative assessment of cardiac fibrosis (Figure 1C) [10]. Among Scn5a+/− mice, the difference in phenotype was maintained over time: each mouse remained in its initial group. Atrial and atrioventricular conduction (as evaluated respectively by P wave and PR interval durations) were similar in the two groups of Scn5a+/− mice and significantly slower than in WT mice (Table 1).
fibrosis led to mean scores of 1.88±0.40 in Scn5a+/− mice with severe phenotype (n = 8; p<0.05 versus WT), 0.60±0.40 in Scn5a+/− mice with mild phenotype (n = 5) and 0.25±0.25 in WT mice (n = 4). No fibrosis was observed in the right ventricular outflow tract region of Scn5a+/− mice with a severe phenotype (not shown). The transcription factors Atf3 and Egr1 were exclusively over-expressed in old Scn5a+/− mice with a severe phenotype (Figure 3B).

Figure 3. Variable levels of fibrosis in Scn5a+/− mice. A. Sirius red staining of ventricle from 85 week-old WT, mildly and severely affected Scn5a+/− mice. Fibrosis appears in red. A score of 0 was attributed to the WT mouse shown, 1 to the mild Scn5a+/− mouse and respectively 2 and 3 to left and right severe Scn5a+/− mice. B. Atf3 and Egr1 mRNA ventricular levels (in arbitrary units) in WT (open bars), mildly (grey bars) and severely (black bars) affected Scn5a+/− mice as a function of age. ***, p<0.001 versus WT and mild.

doi:10.1371/journal.pone.0009298.g003

Ventricular Arrhythmias in Scn5a+/− Mice with a Severe Phenotype and Wider QRS Interval in Symptomatic SCN5A-Related BrS Patients

With aging, Scn5a+/− mice with a severe phenotype had an increased propensity to develop spontaneous ventricular arrhythmias under anesthesia (5/10) in comparison to Scn5a+/− mice with a mild phenotype (0/8; p<0.05) and WT mice (0/12; p<0.05). The severity of the arrhythmias ranged from ventricular extrasystoles to salvos of ventricular tachycardia (Figure 4).

Patients from Nantes database with SCN5A-related BrS were pooled with their counterparts from Amsterdam database. Symptomatic patients, defined as having a clinical history of collapses, syncopes, documented ventricular tachycardia (and/or fibrillation) or aborted sudden death, were characterized by a longer QRS interval than asymptomatic patients (Table 2).

Differential Transcriptional Remodeling between Mild and Severe Phenotype Scn5a+/− Mice

We then investigated the molecular mechanisms potentially accounting for variable penetrance in Scn5a+/− mice. We first hypothesized that molecular remodeling of ion channel gene expression could variably modulate the deficit. Figure 5 shows variations of expression of 46 ion-channel subunits in the ventricles of 10 week-old Scn5a+/− mice relative to their expression in WT littermates. Na,a,1.5 transcripts were similarly down-regulated by almost 50% in Scn5a+/− mice with both mild and severe phenotype, showing that the phenotype heterogeneity was not due to differential transcription of the WT Scn5a allele. Ion channel remodeling in Scn5a+/− mice was limited to 10 genes. Among them, 4 were moderately up-regulated in both groups of Scn5a+/− mice including ankyrin-B, an anchoring protein involved in ion channel (including Nav1.5) and transporter targeting [12,13]. Few genes were differentially regulated among the Scn5a+/− mice. Five were up-regulated only in severely affected Scn5a+/− mice, including genes encoding the Na+ channel α-subunit Na,a,1.3, the T-type Ca2+ channel α-subunit Ca,3.2 and connexin 37. Finally, Kcne1, which encodes KvLQT1 β-subunit, was up-regulated only in mice with mild phenotype. No gene expression (other than Scn5a) was

Figure 4. Cardiac arrhythmias in Scn5a+/− mice with severe phenotype. Representative episodes of spontaneous ventricular arrhythmias recorded in two different Scn5a+/− mice with a severe phenotype under anesthesia. An ECG recorded in an age-matched WT mouse is given for comparison. Scale bar, 500 ms.

doi:10.1371/journal.pone.0009298.g004
significantly down-regulated. The expression of genes encoding pumps and exchangers was not altered.

**Reduced Na\textsubscript{v}1.5 Protein Expression and I\textsubscript{Na} Density in Scn5a\textsuperscript{-/-} Mice with a Severe Phenotype**

We then investigated Na\textsubscript{v}1.5 protein expression. Investigators were blinded to the genotype and phenotype for all the experiments presented below.

In young Scn5a\textsuperscript{-/-} mice, global Na\textsubscript{v}1.5 protein was reduced by 50±4% in ventricular lysates of animals with a severe phenotype versus WT littermates (p<0.001) and by only 21±7% for animals with a mild phenotype (p<0.05 versus severe phenotype, NS versus WT; Figure 6).

At the single cell level, immunofluorescence and confocal imaging experiments demonstrated that Na\textsubscript{v}1.5 staining intensity in intercalated discs was reduced by 42±4% in myocytes from mice with severe phenotype (66 cells from 3 mice; p<0.01 versus WT), but only by 30±2% for mice with mild phenotype (60 cells from 3 mice; p<0.05 versus severe, p<0.01 versus WT; Figure 6C).

Accordingly, the I\textsubscript{Na} density was 28% larger in mice with a mild phenotype (39±7 pA/pF; 18 cells from 4 mice) than in mice with a severe phenotype (46±4 pA/pF; 21 cells from 4 mice; Figure 7) although this difference did not reach significance (p=0.08). For comparison, amplitude of I\textsubscript{Na} in WT mice was 103±7 pA/pF (16 cells from 4 mice). In summary, the three independent techniques indicated a less severe reduction of Na\textsubscript{v}1.5 proteins in mice with a mild phenotype than in mice with a severe phenotype. We also observed a shift towards more positive voltage values of steady-state inactivation and activation curves in Scn5a\textsuperscript{-/-} mice versus WT mice (Table 3). However, no difference was observed among the Scn5a\textsuperscript{-/-} mice.

The decrease in I\textsubscript{Na} induced a moderate (11–15%) non-significant decrease in maximum upstroke velocity (V\textsubscript{max}) of ventricular action potentials in Scn5a\textsuperscript{-/-} mice with mild conduction defects (Figure 8). In contrast, V\textsubscript{max} was significantly decreased by 33–42% in Scn5a\textsuperscript{-/-} mice with a severe phenotype. They also exhibited a significant decrease in action potential amplitude. Other action potential parameters did not differ among the groups.

**Discussion**

The main findings of the present study are: (i) Scn5a\textsuperscript{-/-} mice exhibit variable penetrance of the conduction deficit, as do patients with SCN5A loss-of-function mutations [2,6]; (ii) Scn5a\textsuperscript{-/-} mice with severe ventricular conduction defects, exhibit more myocardial rearrangements with aging than mice with a mild phenotype; (iii) only old mice with a severe phenotype have a markedly reduced conduction reserve and show spontaneous ventricular arrhythmias; (iv) symptomatic BrS patients carrying SCN5A mutations have more pronounced conduction slowing than asymptomatic patients; and (v) the expressivity of the conduction deficit in Scn5a\textsuperscript{-/-} mice is correlated with the expression level of WT functional Na\textsubscript{v}1.5 proteins. Mice with a severe phenotype correspond to the expected haploinsufficient situation since they exhibit ~50% reduction in Na\textsubscript{v}1.5 protein expression whereas Na\textsubscript{v}1.5 reduction in mice with a mild phenotype was less pronounced.

Because distribution of QRS intervals in the Scn5a\textsuperscript{-/-} mouse population is bimodal, we speculate that a compensatory mechanism is active in the mild-phenotype subgroup but inactive in the severe-phenotype subgroup. Such a mechanism governs the severity of the conduction slowing that in turn commands the extent of myocardial rearrangements and fibrosis, confirming our hypothesis of a direct link between ventricular conduction defects and fibrosis [10]. Moreover, that occurrence of large areas of fibrosis in mice with a severe phenotype was preceded by large overexpression of Egr1 and Myf5 in these mice and not in mice with a mild phenotype, which have much less fibrosis, confirms our hypothesis that these transcription factors are involved in myocardial rearrangements [10].

**Molecular Mechanism for Variable Penetrance of the Phenotype in the Mouse**

Because cardiac conduction relies not only on Na\textsuperscript{+} current, but also on cellular coupling and myocardial architecture, different hypotheses could be evoked to explain Scn5a\textsuperscript{-/-} mice phenotypic variability. Variable myocardial rearrangement was not expected to explain phenotypic variability since heterogeneity is already present in young adult mice whereas fibrosis occurs only in older mice [10,11]. We first hypothesized that Na\textsubscript{v}1.5 invalidation could lead to altered expression of other ion channel subunits and of connexins that would vary among mice. Our data indicate that ion channel remodeling was limited to only a few genes. Among them, some were indeed differentially regulated depending on the phenotype severity. However, the contribution of these genes to the phenotype is unlikely since they are either not or weakly expressed in adult mouse ventricle. For instance, the cardiac expression of connexin-37 is restricted to endothelial cells [14]. Overexpression of the mRNA of neuronal Na\textsuperscript{+} channel, Na\textsubscript{v}1.3, and T-type Ca\textsuperscript{2+} channel, Ca\textsubscript{3.2}, may result from a feedback mechanism compensating for the larger conduction deficit of mice with a severe phenotype. In any case, the putative resulting increase in Na\textsuperscript{+} and T-type Ca\textsuperscript{2+} currents would improve conduction and not further deteriorate it. The consequences of KvLQT1 β-subunit (Kvlq1 gene) overexpression in Scn5a\textsuperscript{-/-} mice with a mild phenotype are also difficult to predict. In adult mice, Kvlq1 cardiac expression is restricted to the conduction system [15]. Based on studies performed on Langendorff-perfused hearts showing that targeted disruption of Kvlq1 can alter ventricular conduction velocity [16], one could speculate that Kvlq1 overexpression in Scn5a\textsuperscript{-/-} mice with a mild phenotype could contribute to their shorter QRS interval. However ECG recordings in Kvlq1 knockout mice did not display any alteration of the QRS interval [15], suggesting that this protein is not playing a major role in cardiac impulse propagation.

**Table 2.** ECG characteristics of asymptomatic (n = 84) and symptomatic (n = 24) Scn5a-mutated Brugada patients.

<table>
<thead>
<tr>
<th>Age (yrs)</th>
<th>RR (ms)</th>
<th>P (ms)</th>
<th>PR (ms)</th>
<th>QRS (ms)</th>
<th>QT (ms)</th>
<th>QTc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asymptomatic</td>
<td>41±2</td>
<td>901±18</td>
<td>93±3</td>
<td>191±4</td>
<td>110±2</td>
<td>393±3</td>
</tr>
<tr>
<td>Symptomatic</td>
<td>46±3</td>
<td>915±31</td>
<td>97±4</td>
<td>200±5*</td>
<td>118±4*</td>
<td>401±6</td>
</tr>
</tbody>
</table>

Same abbreviations as in Table 1.

Data are mean ± SEM. *p<0.05 versus asymptomatic patients.

doi:10.1371/journal.pone.0009298.t002
An alternative hypothesis for explaining phenotypic variability was that Scn5a+/− mice had variable reduction in Na⁺ current. Scn5a transcript levels were similar in both groups of Scn5a+/− mice suggesting the same transcriptional regulation of the WT. However, Nav1.5 protein amounts and INa were larger in Scn5a+/− mice with a mild phenotype. The reason for increased expression of Nav1.5 protein in mice with a mild phenotype is as yet unknown. In addition to gene transcription and RNA processing, the expression of functional Nav1.5 proteins will depend on a series of complex interacting processes such as protein synthesis, assembly and post-translational modifications, trafficking to the sarcolemma, anchoring to the cytoskeleton and regulation of endocytosis and degradation, which are yet incompletely understood [17,18].

At first glance, QRS variability might appear unexpectedly large for an inbred mouse strain. It is widely recognized that environmental, investigator-dependent, and time variables can be confounding factors influencing the characterization of mouse

Figure 5. Moderate ionic remodeling in Scn5a+/− mice. Percentage of variation in ventricular expression (Y-axes) of 46 genes encoding ion channel subunits (ch) and connexins (Cx) in Scn5a+/− mice with mild (grey bars; n = 5) and severe (black bars; n = 7) phenotype versus WT mice (n = 10). Sub, subunits. * ** *** p<0.05, p<0.01 and p<0.001 respectively versus WT; † † † † p<0.05 and p<0.001 respectively versus mild phenotype.

doi:10.1371/journal.pone.0009298.g005
phenotypes [19,20]. However, some of these variables are unlikely to explain phenotypic variability of Scn5a+/− mice. Indeed, a single investigator carried out all ECG recordings and analyses. Moreover, since long-term follow-up studies have shown that the mice keep their phenotype severity throughout their life, possible interference with the season or time of the day can be excluded. Finally, and perhaps more importantly, histological studies, immunoblot experiments, patch-clamp and microelectrode recordings were performed under blind conditions.

In both groups of Scn5a+/− mice, the steady-state inactivation and activation and curves were shifted towards more positive voltages than in wild-type mice. To our knowledge, this phenomenon has not described before. It may be speculated that a decrease in the number of sarcolemmal Na+ channels could trigger changes in the expression/interaction of proteins that remain to be indentified altering Nav1.5 biophysical properties. Whatever its mechanism, one consequence of this shift should be that the fraction of Na+ channels available for cardiac depolarization is larger in Scn5a+/− mouse (about 80%) than in wild-type mice (50%).

Potential Implications in PCCD and Brugada Syndrome

We are not aware of long-term individual follow-up data in patients with PCCD that would extend over decades. Comparison between mice and humans is thus difficult to achieve. Our study points to a key similarity between Scn5a+/− mice and loss-of-function SCN5A mutation human carriers. Young Scn5a+/− mice or patients with markedly prolonged QRS interval have a conduction reserve similar to Scn5a+/− mice or patients with mildly prolonged QRS interval, but this reserve fades out with aging. If findings obtained in mouse can be transposed in human, they suggest that depending on the duration of the QRS complex at young age, one may predict the evolution of the conduction disease and requirement for pacemaker in patients with inherited PCCD. Interestingly, in families with SCN5A-related PCCD, the phenotype heterogeneity is high at middle age and some old mutated patients do not develop the disease [2]. In Scn5a+/− mouse, decreased conduction reserve is due to fibrosis and connexin expression remodeling [10,11], which can be considered

Table 3. I_{Na} steady-state activation and inactivation properties of isolated ventricular myocytes from wild-type (WT) and Scn5a+/− mice with mild and severe phenotype.

<table>
<thead>
<tr>
<th>Mouse Phenotype</th>
<th>WT</th>
<th>Mild Scn5a+/−</th>
<th>Severe Scn5a+/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activation</td>
<td>n = 12</td>
<td>n = 13</td>
<td>n = 10</td>
</tr>
<tr>
<td>K (mV/e-fold)</td>
<td>5.9 ± 0.1</td>
<td>6.1 ± 0.2</td>
<td>5.9 ± 0.1</td>
</tr>
<tr>
<td>V1/2 (mV)</td>
<td>-37.6 ± 1.0</td>
<td>-31.2 ± 2.0*</td>
<td>-31.0 ± 1.3*</td>
</tr>
<tr>
<td>Inactivation</td>
<td>n = 10</td>
<td>n = 10</td>
<td>n = 10</td>
</tr>
<tr>
<td>K (mV/e-fold)</td>
<td>6.4 ± 0.2</td>
<td>6.8 ± 0.3</td>
<td>7.0 ± 0.2</td>
</tr>
<tr>
<td>V1/2 (mV)</td>
<td>-77.9 ± 2.5</td>
<td>-68.8 ± 2.5*</td>
<td>-68.0 ± 1.6**</td>
</tr>
</tbody>
</table>

Abbreviations are: n, number of myocytes per group; K, slope factor; V1/2, half-activation or half-inactivation voltage (see methods section for calculation procedure). Data are mean ± sem. *, **, p<0.05 and p<0.01 respectively versus WT (Kruskal-Wallis test, Dunn’s post-test).

doi:10.1371/journal.pone.0009298.g007

doi:10.1371/journal.pone.0009298.g006
structural abnormalities are not detected using routine noninvasive diagnostic tools, fatty replacement, right ventricular fibrosis, myocyte degeneration and apoptosis have been reported [23–25]. Moreover, a boy with compound heterozygosity for two SCN5A mutations exhibited severe degenerative changes in the ventricular conduction system [26]. Taken together, studies in patients and in Scn5a−/− mice strongly suggest that a primary abnormality in Na,1.5 channel may lead to cellular damage. On this basis, arrhythmias may occur when a sufficient degree of myocardial rearrangement has been reached. This would explain why, in the context of an inborn defect, several years (in humans) or months (in mice) might elapse before the first arrhythmic event.

Materials and Methods

Electrics Statement

All the animal experiments were performed in the animal facility (Unité de Therapeutique Experimentale) and the Inserm UMR915 which have been accredited by the French Ministry of Agriculture. Experimental procedures were approved by the regional ethic committee (CREAA – Pays de la Loire).

Studies involving human participants were conducted according to the French and Dutch guidelines for genetic research. All tests that were performed were approved by the medical ethical review committees of the two hospitals involved: Academic Medical Center, Amsterdam, The Netherlands; Centre Hospitale-Universitaire, Nantes, France (CCPRRB des Pays de la Loire). Written informed consent was obtained from the patients.

Electrocardiography in Mouse

All mice with 129/Sv genetic background were bred at l’institut du thorax (Nantes, France) and genotyped by polymerase chain reaction (PCR) as previously described [9]. All experiments were performed on age-matched wild-type (WT) and heterozygous littermates.

Six-lead ECGs were recorded on mice anesthetized with etomidate (25 mg/kg i.p.) and analyzed as previously described [10]. Pharmacological challenge with the Na+ channel blocker ajmaline (20 mg/kg, i.p.) was performed in 10 and >53 week-old mice. QRS interval lengthening was assessed 10 minutes after injection.

ECG Recordings and Clinical Data from Patients with SCN5A Mutations

The study was conducted according to the French and Dutch guidelines for genetic research. All tests that were performed were approved by the medical ethical review committees of the two hospitals involved: Academic Medical Center, Amsterdam, The Netherlands; Centre Hospitale-Universitaire, Nantes, France (CCPRRB des Pays de la Loire). Written informed consent was obtained from the patients.

A first study was aimed at evaluating the effects of ajmaline on conduction defects in patients from the Nantes database carrying SCN5A loss-of-function mutations. The cohort included families with SCN5A mutations leading to Brugada syndrome only and families with SCN5A mutations leading to either progressive cardiac conduction defects (PCCD) or Brugada syndrome [27]. These patients had a median QRS interval duration of 108±12 ms. This value was used as a cut-off to discriminate patients with minor conduction defects (QRS≤108 ms; mild phenotype) or major conduction defects (QRS>108 ms; severe phenotype). Non-mutation carrier siblings served as controls. Two age groups were then distinguished, 6–31 years and 41–61 years. In the 6–31 year-old patient group (mean value: 21±2 years; n = 19), patients with a mild phenotype had a mean QRS duration...
of 98±3 ms (80–108 ms; n = 12) and those with a severe phenotype had a mean QRS duration of 130±7 ms (110–130 ms; n = 7; p<0.001 versus mild). In the older patient group (51±1 years; n = 29), patients with a mild phenotype had a mean QRS interval of 94±2 ms (80–107 ms; n = 11) whereas patients with a severe phenotype had a QRS of 122±4 ms (112–160 ms; n = 12; p<0.001 versus mild). All patients were challenged with ajmaline according to current guidelines [28].

In a second study, clinical data from patients carrying SCN5A mutations leading to Brugada syndrome from the Nantes and Amsterdam databases were pooled (n = 108). This mixed database was used to discriminate symptomatic and asymptomatic patients. Symptomatic patients had a clinical history of collapses, syncope, was used to discriminate symptomatic and asymptomatic patients. Baseline QRS interval duration were

Histology
Cardiac arrest was induced in sedated mice (etomidate, 30 mg/kg i.p.) with intravenous infusion of 10% KCl. After excision, hearts were rinsed in saline solution and fixed by immersion in 10% formalin for 24h. Then, gross transverse sections were cut from the base to the apex and embedded in paraffin. Paraffin sections were stained with hematoxylin-eosin to assess any myocyte necrosis or interstitial inflammation or Sirius red to assess interstitial and scar fibrosis. Serial sections were cut to track the ventricular outflow tract. Semi-quantitative assessment of fibrosis was determined based upon the extent and the number of interstitial fibrosis and scar foci (and scored 0 to 3).

TaqMan Real-Time RT-PCR
For Aft3 and Egr1 genes, Taqman real-time RT-PCR was performed as previously described [10]. The ion channel gene expression patterns of ventricular preparations from WT and Scn5a−/− mice were characterized with TaqMan Low Density Arrays (Applied Biosystems, Foster City, CA) in a two-step RT-PCR process. The genes selected for their cardiac expression encode 68 ion channel α- and auxiliary subunits, 7 proteins involved in calcium homeostasis, 5 transcription factors, specific markers of cardiac regions, vascular vessels, neuronal tissue, fibroblasts, inflammation and hypertrophy, and 4 endogenous control genes used for normalization. First strand cDNA was synthesized from 220 ng of total RNA using the High-Capacity cDNA Archive Kit (Applied Biosystems). Experimental procedure and data analysis were performed as previously described [29].

Protein Isolation and Western Blot
After excision, hearts were rapidly frozen in liquid nitrogen and stored at −80°C. Frozen mouse ventricle was transferred into lysis buffer containing (in mmol/L): tris(hydroxymethyl)aminomethane (TRIS), 50 (pH 7.5); NaCl, 150; ethylenediaminetetra-acetic acid (EDTA), 1; phenylmethylsulfonyl fluoride (PMSF), 1; Complete® protease inhibitor cocktail from Roche Applied Science (Basel, Switzerland). Tissues were then homogenized using a Polytron. Triton Tx-100 was added to a final concentration of 1% and solubilization occurred by rotating for 1 h at 4°C. The soluble fraction from a subsequent 15-min centrifugation at 13,000 g (4°C) was used for the experiments. In order to load each lane of the SDS-page with equivalent amounts of total proteins, the protein concentration of each lysate was measured in triplicate by Bradford assay using a BSA standard curve.

Polyclonal anti-Nav1.5 antibody (ASC-005) was purchased from Alomone (Jerusalem, Israel) and rabbit anti-actin antibody (A 2066) was purchased from Sigma (Buchs, Switzerland). The

Immunofluorescence Experiments on Isolated Cardiomyocytes
Cardiomyocytes were washed with phosphate-buffered saline (PBS, pH 7.4) and fixed in 2% paraformaldehyde (37°C). Cells were blocked/permeabilized in PBS containing 0.075% Triton X-100 and 3% fish oil gelatin, and incubated in primary antibody, (polyclonal Na+,K-1.5 antibody) overnight at 4°C [30]. Following washes (PBS plus 0.1% Triton X-100), cells were incubated in secondary antibody (Alexa 568; Molecular Probes) for 8 hours at 4°C and mounted using Vectashield (Vector). Images were collected on a Zeiss 510 Meta confocal microscope (63 power oil immersion, 1.4 NA, 1.0 Airy Units) using Carl Zeiss Imaging software. Velocity Classification and LSM510 software (Zeiss) were used to measure pixel intensities. The following imaging protocol was adopted. Data were first collected on WT cardiomyocytes to establish a linear imaging protocol. All cardiomyocytes were imaged using an identical imaging protocol (LSM510 ‘re-use’ function to ensure consistency in pinhole diameter, brightness, contrast, and laser intensity). Since Na+,K-1.5 is primarily expressed in the intercalated disc region [31,32], we determined the mean staining intensity for 3 intercalated disc line scans per myocyte. For each group, at least 60 myocytes from 3 different mice were analyzed.

Isolation of Cardiomyocytes and Patch Clamp Experiments
Ventricular myocytes were isolated as previously described [33]. Whole-cell configuration of the patch-clamp technique was used to record INa. Experiments were performed at room temperature (22–23°C), using a VE-2 (Alembic Instruments, Canada) amplifier allowing the recording of large Na+ currents. Pipettes (tip resistance of 1–2 MΩ) were filled with a solution containing (in mmol/L): CsCl, 60; Cesium asparte, 70; CaCl2, 1; MgCl2, 1; HEPES, 10; EGTA, 1; Na2ATP, 5 (pH was adjusted to 7.2 with CsOH). Myocytes were bathed with a solution containing (in mmol/L): NaCl, 10; NMDG-Cl, 120; CaCl2, 2; MgCl2, 1.2; CsCl, 5; HEPES, 10; Glucose 1 (pH was adjusted to 7.4 with CsOH). Holding potentials were −120 mV and IF was densities (pA/pF) were obtained by dividing the peak IF by the cell capacitance. Peak currents were measured during a voltage-clamp protocol.

To quantify the voltage dependence of steady-state activation and inactivation, data from individual cells were fitted with the Boltzmann relationship, y = (Vn) / (1 + exp[(Vn−V)/K]), in which y is the normalized current or conductance, V is the voltage at which half of the available channels are inactivated, K is the slope factor, and Vn is the membrane potential. The voltage dependence of inactivation was determined by measuring current in response to pulses (20 ms) to −20 mV that had been preceded by 300-ms pulses applied in a series of 5-mV incremental voltages from −130 mV to −20 mV.

Microelectrode Studies
Seven wild-type (WT) and 11 Scn5a−/− (6 with a mild phenotype and 5 with a severe phenotype) adult mice of either sex were killed by cervical dislocation. The hearts were quickly removed and immersed in a cool modified Tyrode solution containing (in mmol/L): NaCl, 108; NaHCO3, 25; NaH2PO4, 1.8; KCl, 27;
MgCl₂, 1; CaCl₂, 0.6; glucose, 55 and previously saturated with a 95% O₂-5% CO₂ gas mixture (pH 7.3). Preparations were mounted in a tissue bath chamber, the endocardial surface facing up and superfused with an oxygenated (95% O₂-5% CO₂) Tyrode solution warmed to 37±0.5°C and containing (in mm/L): NaCl, 120; NaHCO₃, 20; (NaH₂PO₄·2H₂O), 1.2; KCl, 5.4; MgCl₂, 1.2; CaCl₂, 1.8; glucose, 10 (pH 7.4). Transmembrane recordings were obtained using standard methods. The tissues were allowed to recover for at least one hour before the experiments started. During this period, they were paced at a frequency of 300 heat per minute (bpm) by bipolar stimulation through Teflon-coated silver wire electrodes. Stimulus pulse width was 1 ms and amplitude was twice diastolic threshold. The preparations were then paced at frequencies increasing from 200 to 800 bpm. Action potentials characteristics were measured at steady state for each frequency.

**Statistical Analysis**

Data are expressed as mean ± SEM. Statistical analysis was performed with Student t-test, Kruskal-Wallis test and one- or two-way analysis of variance by completed by a Tukey test when appropriate. The Fisher Exact test was used for the statistical analysis of proportions of mice with arrhythmias. A value of p<0.05 was considered significant.

**References**