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BASIC SCIENCE

Functional modulation of atrio-ventricular conduction by enhanced late sodium current and calcium-dependent mechanisms in *Scn5a*$^{1798insD/+}$ mice

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Aims

SCN5A mutations are associated with arrhythmia syndromes, including Brugada syndrome, long QT syndrome type 3 (LQT3), and cardiac conduction disease. Long QT syndrome type 3 patients display atrio-ventricular (AV) conduction slowing which may contribute to arrhythmogenesis. We here investigated the as yet unknown underlying mechanisms.

Methods and results

We assessed electrophysiological and molecular alterations underlying AV-conduction abnormalities in mice carrying the *Scn5a*$^{1798insD/+}$ mutation. Langendorff-perfused *Scn5a*$^{1798insD/+}$ hearts showed prolonged AV-conduction compared to wild type (WT) without changes in atrial and His-ventricular (HV) conduction. The late sodium current ($I_{Na,L}$) inhibitor ranolazine (RAN) normalized AV-conduction in *Scn5a*$^{1798insD/+}$ mice, likely by preventing the mutation-induced increase in intracellular sodium ($[Na^+]_i$) and calcium ($[Ca^{2+}]_i$) concentrations. Indeed, further enhancement of $[Na^+]_i$ and $[Ca^{2+}]_i$ by the Na^+/K^+-ATPase inhibitor ouabain caused excessive increase in AV-conduction time in *Scn5a*$^{1798insD/+}$ hearts. *Scn5a*$^{1798insD/+}$ mice from the 129P2 strain displayed more severe AV-conduction abnormalities than FVB/N-*Scn5a*$^{1798insD/+}$ mice, in line with their larger mutation-induced $I_{Na,L}$. Transverse aortic constriction (TAC) caused excessive prolongation of AV-conduction in FVB/N-*Scn5a*$^{1798insD/+}$ mice (while HV-intervals remained unchanged), which was prevented by chronic RAN treatment. *Scn5a*$^{1798insD/+}$-TAC hearts showed decreased mRNA levels of conduction genes in the AV-nodal region, but no structural changes in the AV-node or His bundle. In *Scn5a*$^{1798insD/+}$-TAC mice deficient for the transcription factor *Nfatc2* (effector of the calcium-calcineurin pathway), AV-conduction and conduction gene expression were restored to WT levels.

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Conclusions Our findings indicate a detrimental role for enhanced $I_{Na,L}$ and consequent calcium dysregulation on AV-conduction in *Scn5a*$^{1798insD/+}$ mice, providing evidence for a functional mechanism underlying AV-conduction disturbances secondary to gain-of-function *SCN5A* mutations.

Keywords Atrio-ventricular block/conduction*SCN5A* • mutations • $Na_V1.5$ • Late sodium current • Calcium homeostasis

What's new?

- Enhanced late sodium current and consequent calcium dysregulation in *Scn5a*$^{1798insD/+}$ mice prolongs atrio-ventricular (AV) conduction.
- In *Scn5a*$^{1798insD/+}$ mice, atrio-His rather than His-ventricular conduction is prolonged.
- Atrio-ventricular conduction disturbances in *Scn5a*$^{1798insD/+}$ mice occur in the absence of structural alterations or fibrosis within the AV-nodal or His-Purkinje regions.
- Overall, our findings point to a functional, late sodium current-dependent mechanism underlying AV-conduction disturbances secondary to gain-of-function *SCN5A* mutations.
- Late sodium current inhibition constitutes a potential therapeutic option to restore AV-conduction in long QT syndrome type 3 patients.

Introduction

Mutations in *SCN5A*, the gene encoding the cardiac sodium channel, are associated with a myriad of clinical arrhythmia syndromes including long QT syndrome type 3 (LQT3), Brugada syndrome (BrS), and cardiac conduction disease (CCD). *SCN5A* mutations causing BrS and CCD typically lead to loss of sodium channel function, explaining the associated (atrio-)ventricular conduction slowing.[1] In patients with progressive CCD (or Lev-Lenègre disease), fibrosis formation in the cardiac conduction system with increasing age further slows conduction.[2] Interestingly, atrio-ventricular (AV) conduction slowing (i.e. PR prolongation) is also described in LQT3 patients carrying gain-of-function *SCN5A* mutations associated with an enhanced late sodium current.[3] However, despite its potential detrimental impact on cardiac function and arrhythmogenesis, the mechanisms underlying AV-conduction disturbances secondary to gain-of-function mutations in *SCN5A* remain as yet unclear.

We have previously generated and investigated *Scn5a*$^{1798insD/+}$ mice carrying the mouse homologue of the human mutation *SCN5A*-1795insD. This mutation leads to both decreased peak sodium current (I_{Na}) and increased late sodium current ($I_{Na,L}$) and is associated with a clinical overlap syndrome of BrS, LQT3, and CCD in affected patients.[1] *Scn5a*$^{1798insD/+}$ mice similarly present with (atrio-)ventricular conduction slowing and repolarization abnormalities, the severity of which is dependent on genetic background and varies between two different inbred mouse strains (129P2 and FVB/N).[4] While I_{Na} was decreased to the same extent in *Scn5a*$^{1798insD/+}$ mice from both strains,[4] the increase in $I_{Na,L}$ due to the mutation and the consequent pro-arrhythmic intracellular sodium and calcium dysregulation was found to be larger in the mouse strain presenting the most severe phenotype (i.e. 129P2).[5] Furthermore, we recently observed a modulatory effect of cardiac hypertrophy, a condition associated with altered intracellular calcium signalling, on disease severity in the setting of the mutation.[6] We found that hypertrophy induced by transverse aortic constriction (TAC) caused sudden death and slowing of both ventricular and AV-conduction in *Scn5a*$^{1798insD/+}$ mice, which was attenuated by genetic inhibition of the hypertrophic response and by pharmacological $I_{Na,L}$ blockade. Unexpectedly, TAC induced excessive AV-conduction disturbances in *Scn5a*$^{1798insD/+}$ mice, but the underlying mechanisms were not explored. Therefore, we here investigate in detail the contribution of functional vs. structural remodelling to AV-conduction disturbances in the setting of the *Scn5a*$^{1798insD/+}$ mutation, in particular the role of $I_{Na,L}$ and calcium-dependent mechanisms.

Methods

Methods can be found in the Supplementary material online.

Results

The *Scn5a*$^{1798insD/+}$ mutation is associated with strain-dependent severity in atrio-ventricular conduction slowing

We previously demonstrated that PR-prolongation secondary to the *Scn5a*$^{1798insD/+}$ mutation is dependent on mouse genetic background and is more pronounced in mutant mice of the 129P2 strain compared to FVB/N.[4] We here investigated this in more detail by assessing AV-conduction characteristics in isolated Langendorff-perfused hearts of adult mice (3–5 months) of both strains. During basic atrial stimulation (120 ms cycle length), hearts of *Scn5a*$^{1798insD/+}$ (MUT) mice of the FVB/N strain (FVB/N-MUT) tended to have increased AV-conduction times compared to wild type (WT) hearts of the same strain (FVB/N-WT) (*Figure 1A,B*). The AV-nodal refractory period and the Wenckebach periodicity were similar in FVB/N-WT and FVB/N-MUT (*Figure 1C*). We then assessed AV-conduction parameters in isolated hearts of the 129P2 strain. 129P2-WT hearts displayed longer AV-conduction time compared to FVB/N-WT (*Figure 1A,B*). Moreover, there was a significant increase in AV-conduction time secondary to the *Scn5a*$^{1798insD/+}$ mutation in the 129P2 strain (*Figure 1A,B*). Similarly, the AV-nodal refractory period

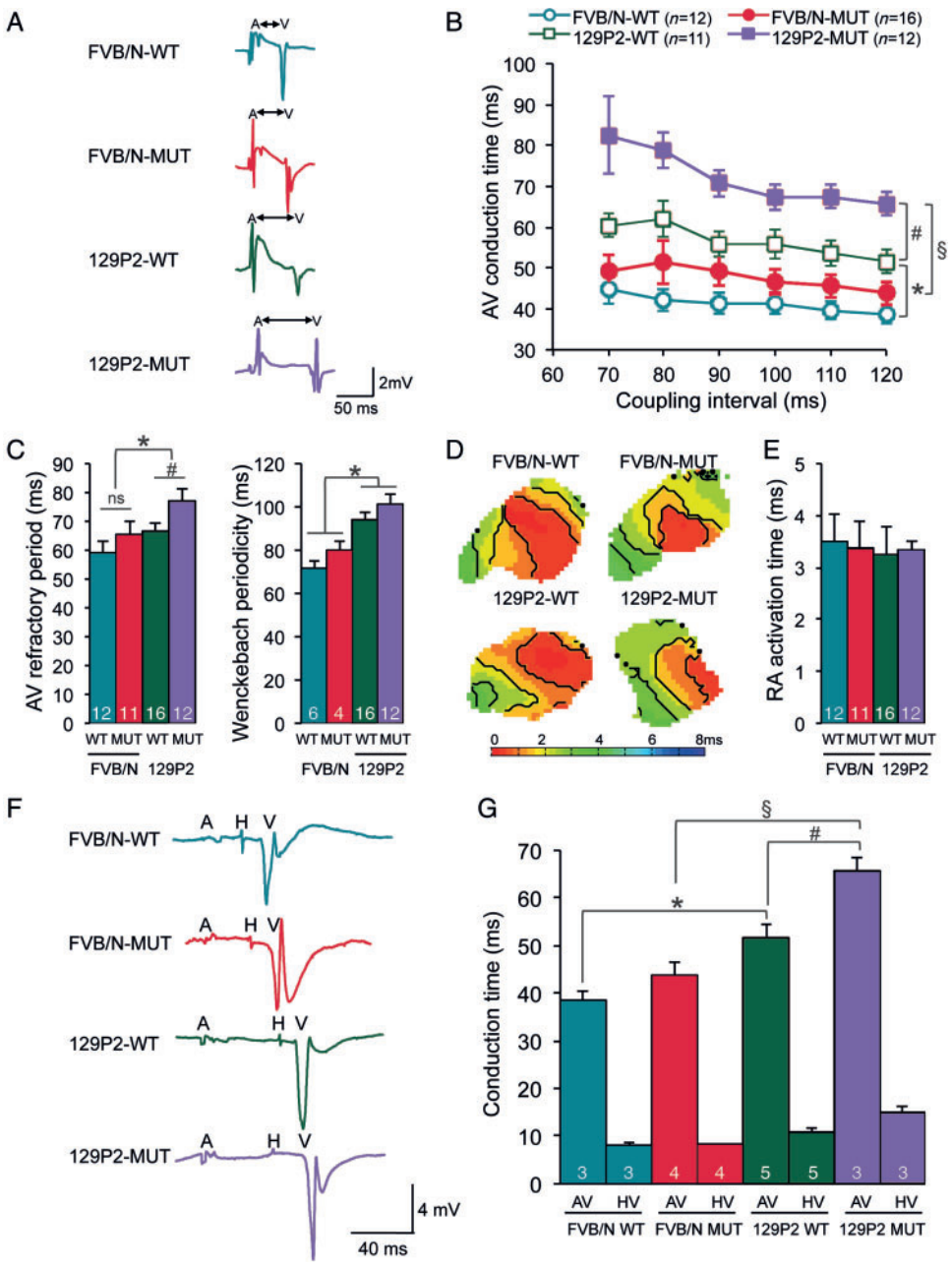

Figure 1 Strain-dependent atrio-ventricular conduction disturbances in *Scn5a*$^{1798insD/+}$ mice. (*A*) Typical examples of atrio-ventricular (AV) conduction time measurements (atrial stimulation 120 ms). (*B*) *Scn5a*$^{1798insD/+}$ (MUT) hearts display longer AV-conduction times at progressively shorter coupling intervals compared to wild type (WT) hearts with the longest AV-conduction times in the 129P2 strain (two-way ANOVA RM: strain × genotype interaction $P = 0.041$; one-way ANOVA RM with LSD *post hoc* testing: $^{*}P = 0.0003$, $^{\#}P = 0.0002$, $^{\S}P = 0.000007$). (*C*) Increased AV-refractory period (two-way ANOVA strain effect $^{*}P = 0.019$, genotype effect $^{\#}P = 0.038$) and Wenckebach periodicity (two-way ANOVA strain effect $^{*}P = 0.00006$) in MUT compared to WT with the longest values in the 129P2 strain. (*D*) Typical examples of right atrial (RA) activation maps. (*E*) Similar RA activation time in FVB/N-WT, FVB/N-MUT, 129P2-WT, and 129P2-MUT. (*F*) Typical examples of His-mapping measurements (AH, atrio-His conduction time; HV, His-ventricular conduction time). (*G*) Similar HV-conduction time in FVB/N-WT, FVB/N-MUT, 129P2-WT, and 129P2-MUT, while AV-conduction time is prolonged in MUT compared to WT, and in 129P2 compared to FVB/N (two-way ANOVA: strain × genotype interaction $P = 0.05$; one-way ANOVA with LSD *post hoc* testing: $^{*}P = 0.0004$, $^{\#}P = 0.001$, $^{\S}P = 0.00002$).

and the Wenckebach periodicity were both significantly longer in the 129P2 strain compared to the FVB/N strain. The AV-nodal refractory period was significantly longer and the Wenckebach periodicity tended to be longer in 129P2-MUT hearts compared to 129P2-WT (*Figure 1C*).

Atrio-ventricular conduction abnormalities in *Scn5a*$^{1798insD/+}$ mice are located in the atrio-ventricular junction

The electrical impulses that traverse the AV-node travel via a number of anatomical structures from the sinus node through the atria, AV-junction, and His-Purkinje system. We first investigated whether (part of) the AV-conduction delay observed in *Scn5a*$^{1798insD/+}$ mice originates from right atrial conduction slowing secondary to the mutation. Right atrial recordings were performed in isolated Langendorff-perfused hearts using optical mapping. Right atrial activation times were similar between all groups (strains and genotypes), indicating that the AV-conduction delay did not originate from the atria (*Figure 1D,E*). We next studied the location of the observed AV-conduction delay within the specialized cardiac conduction system. *Figure 1F* shows electrical recordings illustrating that a His spike was visible before the ventricular signal allowing to distinguish the atrio-His (AH) and His-ventricular (HV) conduction times. At a basic cycle length of 120 ms, we observed no significant differences in HV-conduction time between WT and MUT of the FVB/N strain (*Figure 1F,G*). 129P2-MUT hearts displayed a tendency to increased HV-conduction time compared to WT; this ~4 ms increase in HV-conduction time was however not sufficient to explain the ~14 ms increase in AV-conduction time in 129P2-MUT hearts (*Figure 1F,G*). Thus, the AV-conduction time prolongation observed in *Scn5a*$^{1798insD/+}$ hearts was located predominantly in the supra-Hisian region within the AV-junction.

Atrio-ventricular conduction slowing in *Scn5a*$^{1798insD/+}$ hearts is exacerbated by increasing intracellular sodium and calcium levels

We have previously shown that 129P2 cardiomyocytes have a more pronounced increase in intracellular sodium ($[Na^+]_i$) and calcium ($[Ca^{2+}]_i$) levels secondary to the *Scn5a*$^{1798insD/+}$ mutation compared to FVB/N cardiomyocytes.[5] Since these previous findings were obtained in ventricular myocytes, they do not necessarily reflect the situation in AV-nodal cells. We therefore assessed the effects of ouabain, a cardiac glycoside which increases $[Na^+]_i$ and consequently $[Ca^{2+}]_i$ through inhibition of the plasma membrane Na^+/K^+-ATPase, on AV-conduction in isolated hearts. Upon administration of ouabain, 129P2-MUT hearts developed a higher degree of AV-block (indicated by a slower ventricular rate) with increasing ouabain concentrations compared to 129P2-WT and FVB/N hearts (*Figure 2A,B*). Similarly, a significantly higher number of hearts developed AV-block upon ouabain administration, with all 129P2-MUT hearts developing AV-block following 25 μM ouabain (*Figure 2C*). These findings support a functional role for enhanced $[Na^+]_i$ and $[Ca^{2+}]_i$ in mediating, at least in part, the increased sensitivity of *Scn5a*$^{1798insD/+}$ mice to AV-conduction slowing.

Chronic late sodium current inhibition by ranolazine prevents atrio-ventricular conduction abnormalities in *Scn5a*$^{1798insD/+}$ mice

We have previously shown that the extent of $[Na^+]_i$ and $[Ca^{2+}]_i$ dysregulation in the two mouse strains is mediated by the magnitude of the late sodium current, $I_{Na,L}$.[5] To further investigate the involvement of $I_{Na,L}$ in the $[Na^+]_i$ and $[Ca^{2+}]_i$ dependent AV-conduction abnormalities, the effects of chronic administration of the $I_{Na,L}$ inhibitor ranolazine (RAN) were assessed. Wild type and MUT mice of both strains were fed either control chow (0.03% ketoconazole), or RAN chow (RAN; 0.5% RAN and 0.03% ketoconazole) for the duration of 1 week. Ranolazine chow had no significant effect on AV-conduction time in FVB/N-WT, FVB-MUT, or 129P2-WT, but shortened AV-conduction time in 129P2-MUT hearts (*Figure 2D,E*). In fact, RAN reduced the AV-conduction time observed in the 129P2-MUT hearts to 129P2-WT levels. Similarly, RAN chow had a tendency to shorten AV-refractory period and Wenckebach periodicity only in 129P2-MUT compared to 129P2-MUT without RAN treatment (*Figure 2F,G*). These results confirm a functional role for $I_{Na,L}$ in mediating AV-conduction abnormalities in 129P2-MUT mice.

Atrio-ventricular conduction time prolongation in FVB/N-*Scn5a*$^{1798insD/+}$ mice subjected to pressure overload

While the mutation induced a robust phenotype in the 129P2 strain, FVB/N-MUT mice only displayed a mild phenotype. On the other hand, our previous work has shown that FVB/N-MUT mice subjected to chronic pressure overload developed severe AV-conduction abnormalities and sudden cardiac death.[6] Here, we investigated in more detail the characteristics of the AV-conduction abnormalities in FVB/N mice subjected to transverse aortic constriction (TAC), a procedure that leads to cardiac hypertrophy. During baseline atrial stimulation at 120 ms, isolated Langendorff-perfused MUT-TAC hearts displayed significantly longer AV-conduction times compared to WT-TAC or MUT-Sham hearts, while TAC had no effect on AV-conduction time in WT hearts (*Figure 3A,B*). This was further exacerbated at shorter coupling intervals with three MUT-TAC hearts displaying AV-block already at coupling intervals of 90 ms (*Figure 3B*). Both AV-refractory period and Wenckebach periodicity were significantly increased in MUT-TAC hearts compared to MUT-Sham and WT-TAC (*Figure 3C*). Although decreased expression of *Scn5a*, *Gja5*, and *Gja1* was observed in right atrial tissue after TAC (Supplementary material online, *Figure S1*), right atrial activation times were similar in all groups, demonstrating that TAC did not induce atrial conduction delay in either WT or MUT mice (*Figure 3D,E*). Moreover, we observed no significant differences in HV-conduction time between WT-Sham, MUT-Sham, WT-TAC, and MUT-TAC hearts when stimulated at 120 ms (*Figure 3F,G*). Thus, the prolonged AV-conduction delay in MUT-TAC mice occurred in the supra-Hisian region within the AV-junction.

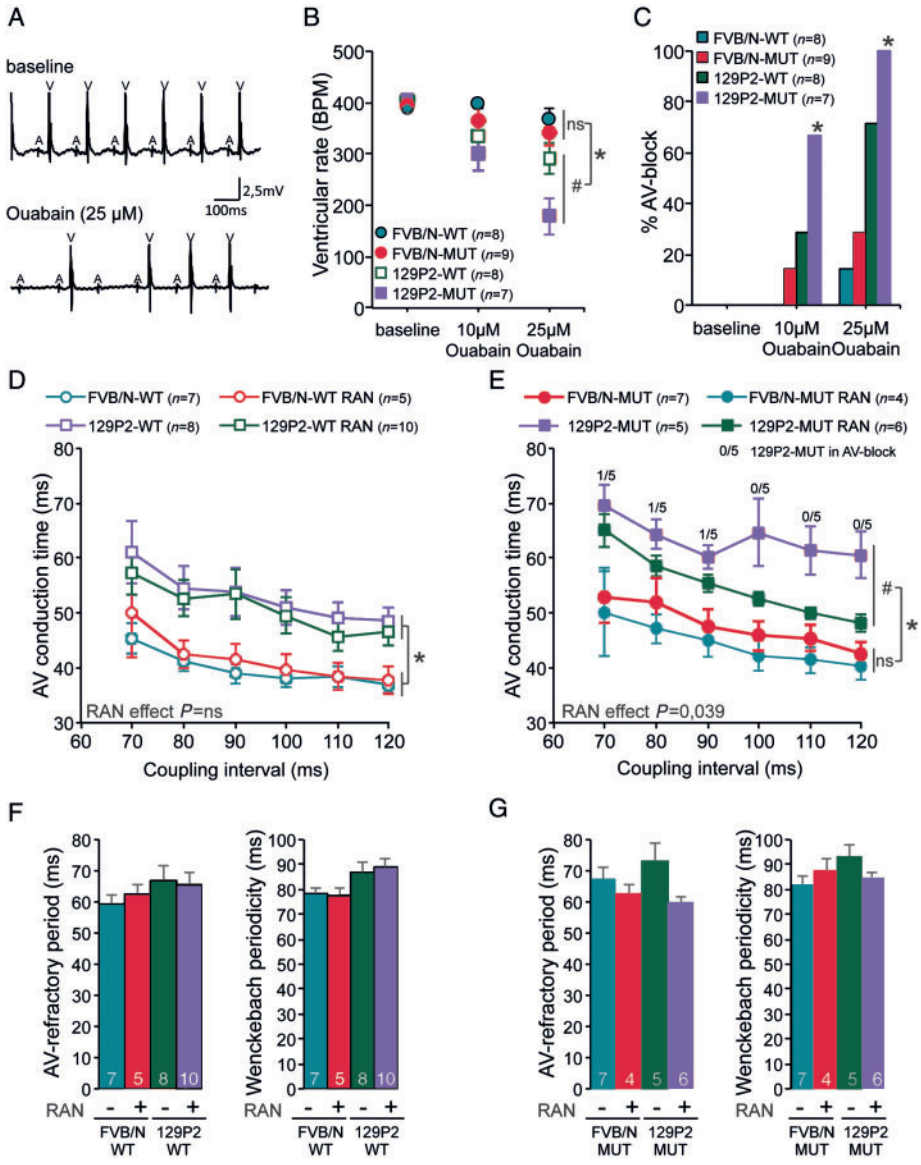

Figure 2 Modulation of atrio-ventricular conduction by intracellular sodium and calcium levels and $I_{Na,L}$ (*A*) Typical examples of atrio (A)-ventricular (V) measurements in the absence or presence of ouabain (25 µM) in a 129P2-MUT heart (atrial stimulation 150 ms). (*B*) Slower ventricular rate [beats per minute (BPM)] in 129P2-MUT hearts in the presence of 10 µM and 25 µM ouabain compared to 129P2-WT, FVB/N-MUT, and FVB/N-WT (two-way ANOVA for 25 µM strain effect $^{*}P = 0.002$, genotype effect $^{\#}P = 0.03$). (*C*) Increased occurrence of AV-block with increasing ouabain concentrations; all 129P2-MUT hearts display AV-block at 25 µM ouabain (χ^2 for 10 µM $^{*}P = 0.0124$ and for 25 µM $^{*}P = 0.0051$). (*D*) Ranolazine (RAN) did not affect AV-conduction time in 129P2-WT or FVB/N-WT (two-way ANOVA RM strain effect $^{*}P = 0.004$). (*E*) RAN shortened AV-conduction time in 129P2-MUT (two-way ANOVA RM strain effect $^{*}P = 1 \times 10^{-5}$, RAN effect $^{\#}P = 0.039$). (*F*) and (*G*) RAN had a tendency to shorten AV-refractory period and Wenckebach periodicity in 129P2-MUT only.

Atrio-ventricular conduction delay in FVB/N-*Scn5a*$^{1798insD/+}$ transverse aortic constriction mice occurs in the absence of structural abnormalities

Cardiac hypertrophy secondary to TAC may be associated with development of fibrosis, which when affecting the specialized conduction system may lead to delayed AV-conduction. However, detailed histological assessment in Heidenhain trichrome stained sections revealed no fibrosis or other degenerative changes within the atrium or AV-node region of either WT-TAC or MUT-TAC hearts (*Figure 4A*). Some mild fibrosis was observed at the bifurcating bundle and proximal bundle branches, but here a similar pattern was observed in both WT-TAC and MUT-TAC hearts (*Figure 4B*). Thus, AV conduction was impaired in structurally normal AV-nodes of *Scn5a*$^{1798insD/+}$ hearts after 2 weeks of pressure overload, in line with a functional mechanism as the underlying cause.

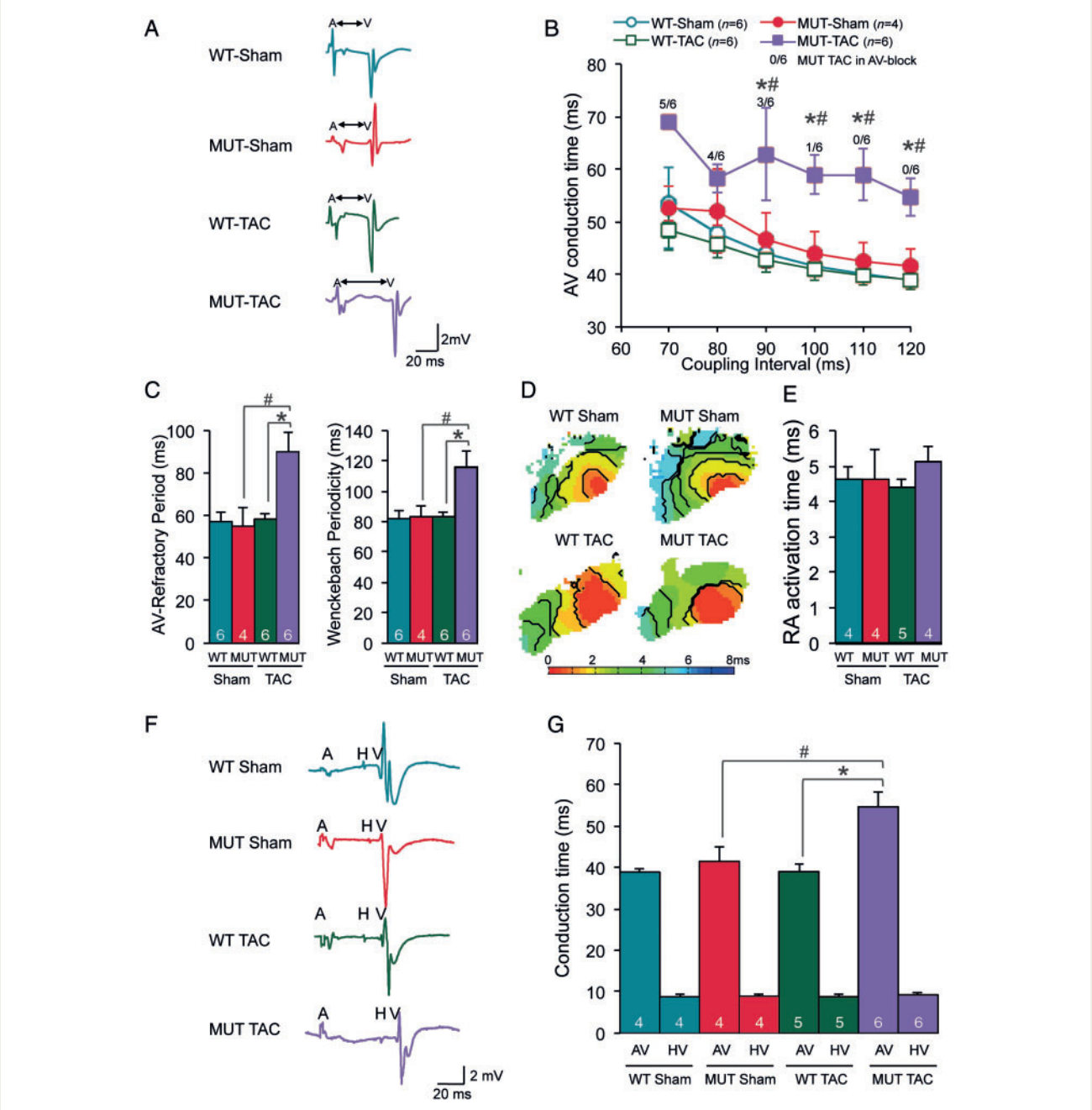

Figure 3 Cardiac hypertrophy prolongs atrio-ventricular conduction in *Scn5a*$^{1798insD/+}$ mice. (*A*) Typical examples of atrio-ventricular (AV) measurements (atrial stimulation 120 ms). (*B*) Increased AV-conduction time in MUT-TAC compared to WT-Sham, MUT-Sham, and WT-TAC (two-way ANOVA per coupling interval: genotype × procedure interaction, all significant; one-way ANOVA per coupling interval with LSD *post hoc* testing $^{*}P < 0.05$ MUT-TAC vs. WT-TAC, $^{\#}P < 0.05$ MUT-TAC vs. MUT-Sham). (*C*) Increased AV-refractory period (two-way ANOVA: genotype × procedure interaction $P = 0.014$; one-way ANOVA with LSD *post hoc* testing: $^{*}P = 0.001$, $^{\#}P = 0.002$) and Wenckebach periodicity (two-way ANOVA: genotype × procedure interaction $P = 0.02$; one-way ANOVA with LSD *post hoc* testing: $^{*}P = 0.008$, $^{\#}P = 0.015$) in MUT-TAC compared to WT-Sham, MUT-Sham, and WT-TAC. (*D*) Typical examples of right atrial (RA) activation maps. (*E*) Similar RA activation time in WT-Sham, MUT-Sham WT-TAC, and MUT-TAC. (*F*) Typical example of His-mapping measurements (AH, atrio-His conduction time; HV, His-ventricular conduction time). (*G*) Similar HV-conduction time in WT-Sham, MUT-Sham WT-TAC, and MUT-TAC, while AV-conduction time is prolonged in MUT-TAC only (two-way ANOVA: genotype × procedure interaction $P = 0.02$, one-way ANOVA with LSD *post hoc* testing $^{*}P = 0.0002$, $^{\#}P = 0.003$).

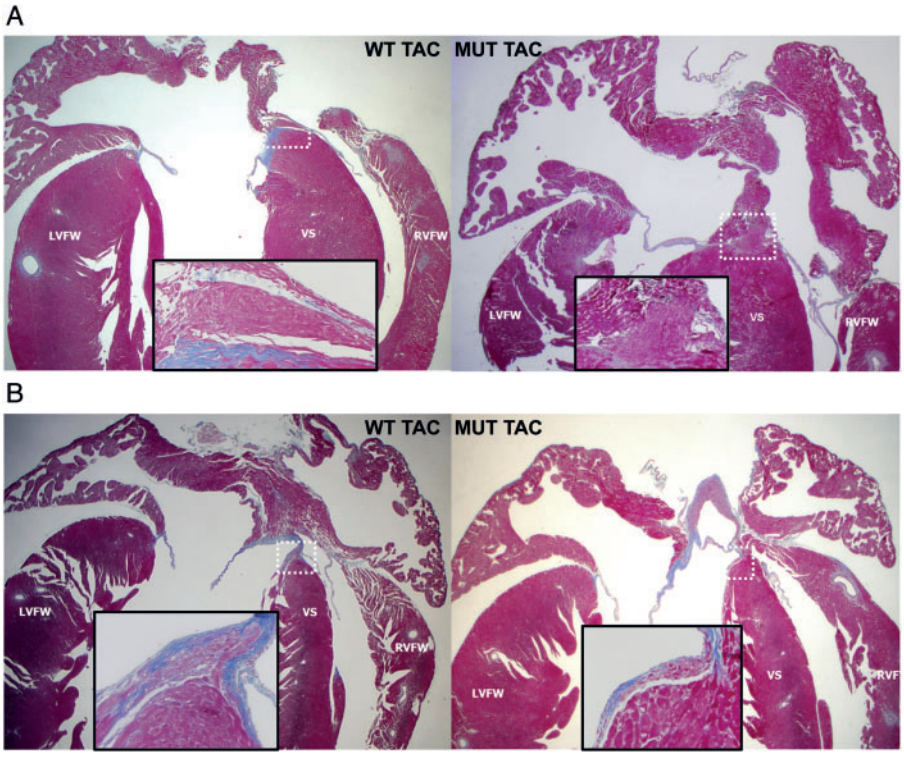

Figure 4 Absence of structural abnormalities in *Scn5a*$^{1798insD/+}$ mice subjected to TAC. (*A*) Absence of fibrosis (stained by Heidenhain trichrome, in blue) in the AV-node region of WT-TAC and MUT-TAC. (*B*) Presence of fibrosis in the bifurcating bundles of both WT-TAC and MUT-TAC. AV, atrio-ventricular; LVFW, left ventricular free wall; RVFW, right ventricular free wall; TAC, transverse aortic constriction; VS: ventricular septum; WT, wild type.

Chronic late sodium current inhibition or genetic inhibition of the hypertrophic response normalizes atrio-ventricular conduction in FVB/N-*Scn5a*$^{1798insD/+}$ mice subjected to transverse aortic constriction

Given that cardiac hypertrophy is associated with enhanced $I_{Na,L}$ and alterations in calcium signalling,[7,8] we investigated in more detail the previously observed effect of inhibition of $I_{Na,L}$ by RAN on AV-conduction in *Scn5a*$^{1798insD/+}$ mice subjected to pressure overload.[6] While RAN chow had no effect in WT-TAC, it prevented AV-block and shortened AV-conduction time in MUT-TAC at progressively shorter coupling intervals compared to MUT-TAC mice on control chow (*Figure 5A,B*). Atrio-ventricular nodal refractory period and Wenckebach periodicity were also significantly shortened in MUT-TAC treated with RAN compared to control (*Figure 5C*). No differences were observed in Sham hearts (Supplementary material online, *Table S1*). In order to investigate the acute effects of $I_{Na,L}$ blockade, we exposed Langendorff-perfused WT-TAC and MUT-TAC hearts to 10 μM RAN for a duration of 15 min, which had no effect on AV-conduction time nor on AV-refractory period in either group (Supplementary material online, *Figure S2*). Therefore, the shortening of AV-conduction time in MUT-TAC induced by chronic exposure to $I_{Na,L}$ blockade is most likely mediated by a chronic downstream mechanism. Chronic pressure overload may lead to activation of transcriptional calcium-dependent pathways such as the pro-hypertrophic calcineurin-Nfat pathway.[7] The main downstream effector of this pathway is Nuclear Factor of Activated T cells (Nfatc2), a transcription factor involved in pathologic cardiac remodelling during the development of cardiac hypertrophy.[7] We have previously shown that deletion of *Nfatc2* attenuated AV-conduction delay in MUT-TAC mice (MUT-*Nfatc2*$^{-/-}$-TAC).[6] Investigating this in more detail, we found that MUT-TAC hearts with intact expression of *Nfatc2* (MUT-*Nfatc2*$^{+/+}$-TAC) had prolonged AV-conduction time and increased AV-block at progressively shorter coupling intervals compared to WT-*Nfatc2*$^{+/+}$-TAC, while MUT-*Nfatc2*$^{-/-}$-TAC hearts displayed similar AV-conduction properties to WT-*Nfatc2*$^{-/-}$-TAC and WT-*Nfatc2*$^{+/+}$-TAC (*Figure 5D,E*). Moreover, the TAC induced increase in AV-refractory period and Wenckebach periodicity in MUT-*Nfatc2*$^{+/+}$ was abrogated in MUT-*Nfatc2*$^{-/-}$ hearts (*Figure 5F*). No differences were observed in Sham hearts (Supplementary material online, *Table S1*). Taken together, the rescue of TAC-induced AV-conduction abnormalities by either RAN or *Nfatc2* deletion attests to a functional role for enhanced $I_{Na,L}$ and calcium-dependent signalling in modulating AV-conduction in *Scn5a*$^{1798insD/+}$ mice in the setting of cardiac hypertrophy.

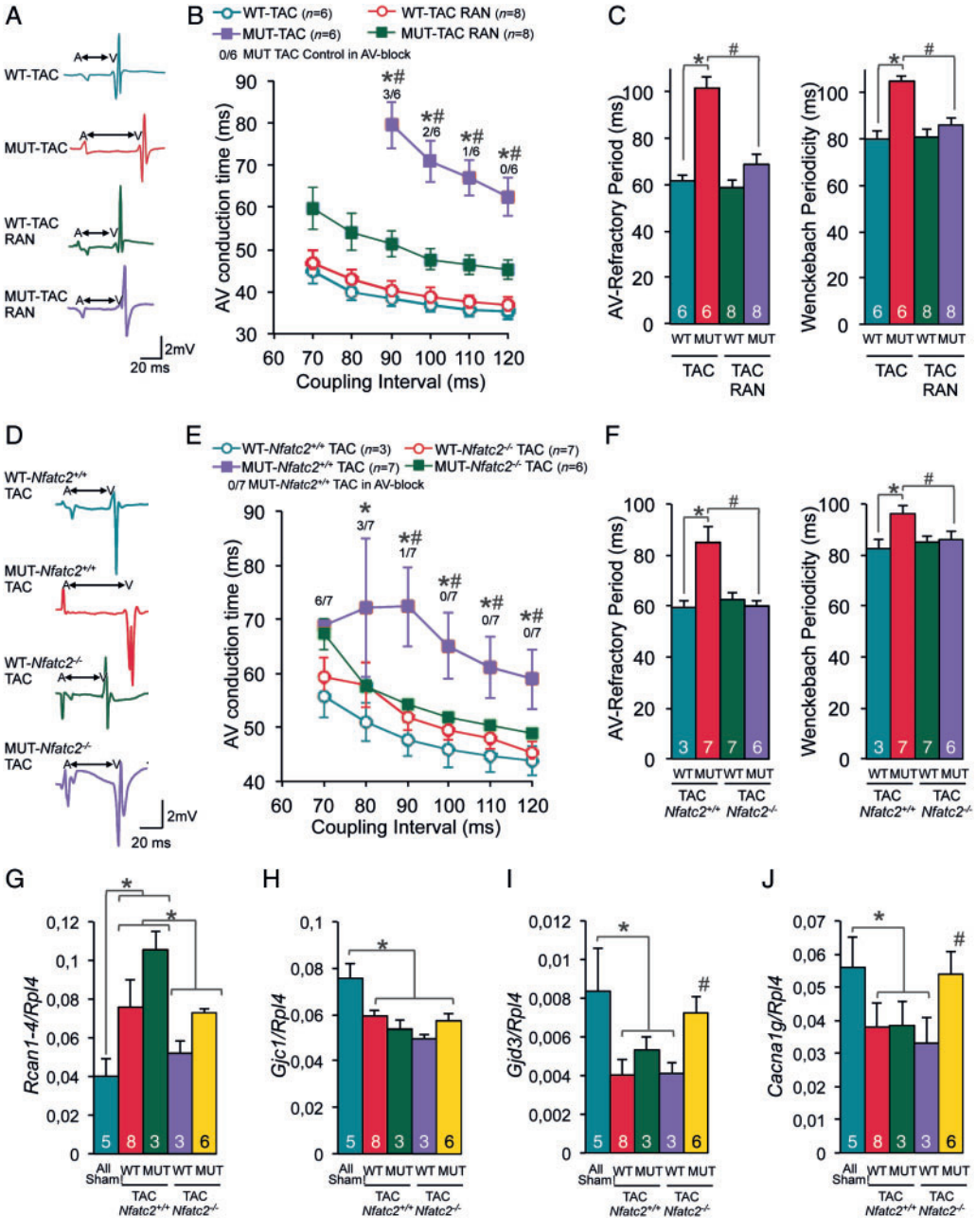

Figure 5 Normalization of AV-conduction time in *Scn5a*$^{1798insD/+}$ TAC mice subjected chronic late sodium current inhibition and in *Scn5a*$^{1798insD/+}$-*Nfatc2*$^{-/-}$ TAC mice. (*A*) Typical examples of atrio-ventricular (AV) measurements (atrial stimulation 120 ms). (*B*) Decreased AV-conduction time in MUT-TAC treated with ranolazine (RAN) chow compared to MUT-TAC treated with control chow (two-way ANOVA per coupling interval: genotype × treatment interaction, all significant; one-way ANOVA per coupling interval with LSD *post hoc* testing $^{*}P < 0.05$ MUT-TAC vs. WT-TAC, $^{\#}P < 0.05$ MUT-TAC vs. MUT-TAC RAN). (*C*) Decreased AV-refractory period (two-way ANOVA: genotype × treatment interaction $P = 0.001$; one-way ANOVA with LSD *post hoc* testing $^{*}P = 9 \times 10^{-7}$, $^{\#}P = 6 \times 10^{-6}$) and Wenckebach periodicity (two-way ANOVA: genotype × treatment interaction $P = 0.006$; one-way ANOVA with LSD *post hoc* testing $^{*}P = 0.00004$, $^{\#}P = 0.001$) in MUT-TAC treated with ranolazine (RAN) chow compared to MUT-TAC treated with control chow. (*D*) Typical examples of atrio-ventricular (AV) measurements (atrial stimulation 120 ms). (*E*) Decreased AV-conduction time in MUT-*Nfatc2*$^{-/-}$-TAC compared to MUT-*Nfatc2*$^{+/+}$-TAC (two-way ANOVA per coupling interval: treatment × genotype interaction, all significant; one-way ANOVA per coupling interval with LSD *post hoc* testing $^{*}P < 0.05$ MUT-*Nfatc2*$^{+/+}$-TAC vs. WT-TAC, $^{\#}P < 0.05$ MUT-*Nfatc2*$^{+/+}$-TAC vs. MUT-*Nfatc2*$^{-/-}$-TAC). (*F*) Decreased AV-refractory period (two-way ANOVA: genotype × genotype interaction $P = 9 \times 10^{-5}$; one-way ANOVA with LSD *post hoc* testing $^{*}P = 8 \times 10^{-6}$, $^{\#}P = 4 \times 10^{-6}$) and Wenckebach periodicity (two-way ANOVA: genotype × genotype interaction $P = 0.03$; one-way ANOVA with LSD *post hoc* testing $^{*}P = 0.01$, $^{\#}P = 0.04$) in MUT-*Nfatc2*$^{-/-}$-TAC compared to MUT-*Nfatc2*$^{+/+}$-TAC. (*G–J*) Atrio-ventricular-nodal expression of *Rcan1-4*, *Gjc1* (Cx45), *Gjd3* (Cx30.2), *Cacna1g* (Cav3.1), in WT, MUT, WT-*Nfatc2*$^{-/-}$, and MUT-*Nfatc2*$^{-/-}$ (one-way ANOVA with LSD *post hoc* testing $^{*}P < 0.05$, $^{\#}P < 0.05$ MUT-*Nfatc2*$^{+/+}$-TAC vs. MUT-*Nfatc2*$^{-/-}$-TAC).

Calcium-dependent control of atrio-ventricular junction gene expression in *Scn5a*$^{1798insD/+}$ transverse aortic constriction mice

While the transcriptional effects of the calcineurin-Nfat pathway may be similar in various parts of the heart, the functional consequences may differ according to the molecular make-up of these regions. This pathway may affect AV-conduction by regulating transcription of various genes in the AV-node. To investigate this hypothesis in more detail, we micro-dissected the AV-nodal region (AVN) and isolated total RNA from WT and MUT mice (either on a *Nfatc2*$^{+/+}$ or a *Nfatc2*$^{-/-}$ background) subjected to TAC (Sham mice were used as controls) and assessed the expression level of genes involved in AV-conduction. mRNA expression levels of *Rcan1-4*, a known direct target of the calcineurin-Nfat pathway, were significantly lower in AVN from MUT-*Nfatc2*$^{-/-}$-TAC than MUT-*Nfatc2*$^{+/+}$-TAC (*Figure 5G*), suggesting a lower degree of induction of the calcineurin pathway after TAC in mice on the *Nfatc2*$^{-/-}$ background. Transverse aortic constriction induced a significant reduction in mRNA expression levels of genes essential for AV-conduction, including connexin45 (Cx45; gene *Gjc1*), Cx30.2 (gene *Gjd3*), and T-type calcium channel 3.1 (Cav3.1; gene *Cacna1g*) (*Figure 5H–J*). Importantly, levels of AVN-specific genes *Gjd3* and *Cacna1g* were higher in MUT-*Nfatc2*$^{-/-}$-TAC compared to MUT-*Nfatc2*$^{+/+}$-TAC, and similar to the Sham mice, thus potentially contributing to the restored AV-conduction observed in MUT-*Nfatc2*$^{-/-}$-TAC mice (*Figure 5I,J*).

Discussion

Our current findings point to a dynamic relation between chronically enhanced $I_{Na,L}$, subsequent $[Na^+]_i$ and $[Ca^{2+}]_i$ dysregulation, and AV-conduction delay in *Scn5a*$^{1798insD/+}$ mice. Our results moreover provide further evidence for a modulatory role for the calcium-dependent calcineurin-Nfat signalling pathway in mediating AV-conduction in *Scn5a*$^{1798insD/+}$ mice subjected to pressure overload. Importantly, the observed AV-conduction delay occurred in the absence of structural alterations in the AV-node. Taken together, these observations attest to a functional mechanism underlying AV-conduction disturbances secondary to *SCN5A* mutations associated with gain of sodium channel function.

Modulation of atrio-ventricular conduction in *Scn5a*$^{1798insD/+}$ mice by enhanced $I_{Na,L}$ and calcium dysregulation

Multiple observations in the current study support the concept that prolongation of AV-conduction in *Scn5a*$^{1798insD/+}$ mice is mediated by $[Na^+]_i$ and $[Ca^{2+}]_i$ dysregulation secondary to enhanced $I_{Na,L}$. Firstly, hearts from 129P2-*Scn5a*$^{1798insD/+}$ mice displayed more pronounced AV-conduction disturbances compared to FVB/N-*Scn5a*$^{1798insD/+}$ hearts, and we have previously shown that the mutation leads to a larger increase in $I_{Na,L}$ and consequently higher levels of $[Na^+]_i$ and $[Ca^{2+}]_i$ in cardiomyocytes from 129P2-*Scn5a*$^{1798insD/+}$ compared to FVB/N-*Scn5a*$^{1798insD/+}$ mice.[5] Moreover, 129P2-*Scn5a*$^{1798insD/+}$ hearts developed more severe prolongation of AV-conduction time and a higher degree of AV-block following ouabain administration; ouabain is known to increase $[Na^+]_i$ and $[Ca^{2+}]_i$ and, consequently, to prolong AV-conduction time and to cause AV-block in dogs.[9] Finally, the $I_{Na,L}$ blocker RAN, which we have previously shown to prevent $[Na^+]_i$ and $[Ca^{2+}]_i$ dysregulation in ventricular *Scn5a*$^{1798insD/+}$ cardiomyocytes,[5] restored normal AV-conduction in *Scn5a*$^{1798insD/+}$ mice from the 129P2 strain. A direct role of $[Na^+]_i$ and $[Ca^{2+}]_i$ homeostasis would ideally be assessed within AV-nodal cells, but their isolation from mouse hearts is extremely challenging with a low yield. Our results from Langendorff-perfused hearts nevertheless clearly indicate a functional role for $I_{Na,L}$-mediated Na^+/Ca^{2+}-dysregulation in modulation of AV-conduction abnormalities secondary to gain-of-function *SCN5A* mutations.

Exacerbated atrio-ventricular-conduction time in *Scn5a*$^{1798insD/+}$ mice following transverse aortic constriction: role of enhanced $I_{Na,L}$, calcium dysregulation and calcium-dependent signalling

The functional role of enhanced $I_{Na,L}$ and calcium dysregulation in mediating AV-conduction time was further evidenced by our observations in *Scn5a*$^{1798insD/+}$ mice subjected to transverse aortic constriction (TAC). Apart from inducing cardiac hypertrophy, TAC is also known to enhance $I_{Na,L}$ and subsequently increase pro-arrhythmic $[Ca^{2+}]_i$.[8] In FVB/N-WT mice, the TAC procedure (or its relative short duration of 2 weeks) and its ionic consequences were not sufficient to induce any AV-conduction disturbances. However, FVB/N-*Scn5a*$^{1798insD/+}$, which already have a (modestly) increased $I_{Na,L}$ and $[Ca^{2+}]_i$,[5] developed prolonged AV-conduction time and AV-block following TAC. In these hearts, no fibrosis was observed in the supra-Hisian region, suggesting a functional rather than a structural underlying mechanism. Indeed, chronic treatment with RAN restored AV-conduction in FVB/N-*Scn5a*$^{1798insD/+}$ mice subjected to TAC, providing evidence for a mechanistic role involving $I_{Na,L}$ enhancement. Increased $I_{Na,L}$ may in itself affect AV-conduction by prolonging refractoriness and action potential duration. However, acute RAN administration did not ameliorate the TAC-induced AV-conduction abnormalities in FVB/N-*Scn5a*$^{1798insD/+}$ hearts, indicating that the underlying mechanism involves a chronic effect of enhanced $I_{Na,L}$ and associated activation of calcium signalling pathways. Here, increased $[Ca^{2+}]_i$ in cardiomyocytes may depress AV-conduction directly[9] (although the exact mechanisms are unclear), or indirectly via calcium-dependent signalling. The calcineurin-Nfat pathway is activated by sustained elevated $[Ca^{2+}]_i$; when calcineurin becomes activated it dephosphorylates its transcriptional effector Nfat (nuclear factor of activated T cells) in the cytoplasm.[7] This results in translocation of Nfat to the nucleus where it interacts with other transcription factors to modulate gene expression.[7] Mice with a chronically activated calcineurin pathway develop cardiac hypertrophy and display premature sudden death as well as AV-conduction abnormalities with markedly prolonged PR-intervals and a high degree of AV-block.[7,10] Our observation that TAC induced overt AV-conduction disturbances in *Scn5a*$^{1798insD/+}$ mice, but not in WT, suggests that the elevated $[Ca^{2+}]_i$ caused by the mutation sensitizes hearts to calcineurin pathway activation. Indeed, AV-conduction abnormalities in

Scn5a$^{1798insD/+}$ mice were also rescued by genetic inhibition of *Nfatc2* thus providing unequivocal evidence for a functional role of the calcineurin-Nfat pathway in modulating AV-conduction in *Scn5a*$^{1798insD/+}$ mice subjected to pressure overload.

Transcriptional regulation in atrio-ventricular nodal region and atrio-ventricular conduction

Cardiac conduction slowing may stem from multiple pathophysiological mechanisms affecting cell–cell electrical coupling, including ion channel and gap junction remodelling, and fibrosis, which may not necessarily be mutually exclusive. Although enhanced calcineurin-Nfat signalling is known to lead to the development of fibrosis,[7] we did not detect fibrosis or other degenerative changes within the AV-nodal region of either wild type or *Scn5a*$^{1798insD/+}$ mice subjected to TAC, suggesting an underlying functional component that is mediated through ion channel and/or gap junction remodelling. Indeed, we found that the expression levels of connexin45 (Cx45) and conduction system-specific connexin30.2 (Cx30.2) and calcium channel 3.1 (Cav3.1) were downregulated in the AV-node region after TAC. In line with this, mice with conduction system-specific deletion of both Cx45 and Cx30.2 have previously been shown to display a significant prolongation of AV-conduction time and AV-block proximal to the His-bundle, while deletion of Cx45 alone only mildly prolonged AV-conduction.[11] In addition, Cav3.1$^{-/-}$ mice displayed prolongation of the AH-interval without affecting right atrial impulse propagation,[12] in accordance with our observation that atrial activation times and HV-interval duration are not affected in *Scn5a*$^{1798insD/+}$mice and after TAC. Atrial conduction was overall unaffected by TAC despite a decrease in mRNA expression of *Scn5a*, *Gja5*, and *Gja1* in right atrial tissue. This may be due to the fact that expression of these genes needs to be substantially reduced for conduction to be affected,[13,14] particularly in the absence of structural remodelling. Moreover, higher expression of $Na_V1.5$ has been reported in murine atrial compared to ventricular tissue,[15] suggesting a potentially larger conduction reserve in atria. In any case, the unchanged right atrial activation time indicates that alterations in atrial conduction do not contribute (or at most only marginally) to the observed AV-conduction abnormalities following TAC. Cx45 expression levels were unchanged in the AV-nodes of *Scn5a*$^{1798insD/+}$-*Nfatc2*$^{-/-}$-TAC mice compared to *Scn5a*$^{1798insD/+}$-*Nfatc2*$^{+/+}$-TAC mice, suggesting that normalization of Cx45 is not required for AV-conduction to be restored. However, Cav3.1 and Cx30.2 levels were both normalized by genetic deletion of *Nfatc2*$^{-/-}$, indicating that changes in transcriptional activity mediated by calcineurin-Nfat pathway activation likely contributed to the observed AV-conduction disturbances in *Scn5a*$^{1798insD/+}$ mice subjected to TAC.

Potential clinical relevance and therapeutic considerations in long QT syndrome type 3 patients

Treatment of LQT3 patients typically includes beta-blockers with adjunct ICD implantation in patients considered to be at high risk for serious cardiac events. More recently, $I_{Na,L}$ inhibition has been suggested as a potential beneficial (additive) therapy.[16] While AV-conduction disturbances have been reported in LQT3 patients,[3] their relevance for disease outcome are as yet unknown. Excessively prolonged AV-conduction may ultimately lead to ventricular remodelling (including cardiac hypertrophy) and associated alterations in repolarization, which may further predispose to arrhythmias.[17] In addition, AV-block and a consequent low ventricular rate could increase the likelihood of pause- or bradycardia-related QT-prolongation and Torsades de Pointes arrhythmias. Whether AV-conduction disturbances also contribute to the increased susceptibility to dilated cardiomyopathy observed in some *SCN5A* mutation carriers remains speculative. Therefore, prevention of AV-conduction abnormalities by inhibition of $I_{Na,L}$ as demonstrated in *Scn5a*$^{1798insD/+}$ mice in the current study may have an additional benefit. However, most $I_{Na,L}$ inhibitors also affect peak sodium current to some extent thereby potentially worsening conduction abnormalities, particularly with increasing age.[16] Previous studies aiming to pharmacologically shorten the QT interval in LQT3 patients with sodium channel inhibitors have demonstrated variable effects on the PR-interval, but study sizes were small and not specifically designed to assess effects on AV-conduction.[18] Newly developed, more selective $I_{Na,L}$ inhibitors may prove more suitable.[16] Future studies should address their potential beneficial effects on both ventricular repolarization and AV-conduction in LQT3.

Potential limitation of the *Scn5a*$^{1798insD/+}$ mouse model

In addition to enhancing $I_{Na,L}$, the *Scn5a*$^{1798insD/+}$ mutation is also known to decrease peak I_{Na}. In the current study, we investigated in detail the chronic effect of enhanced $I_{Na,L}$ and consequent $[Ca^{2+}]_i$ dysregulation and calcium-dependent signalling on AV-conduction. A similar exploration of the role of the mutation-induced decreased peak I_{Na} in mediating part of the AV-conduction slowing was not possible due to the lack of (pharmacological) tools to specifically increase peak I_{Na} *in vivo*. *Scn5a* is expressed in the transitional zone of the AV-node, albeit to a lesser extent than in atrial tissue.[19] Nevertheless, it has been reported that this low level of *Scn5a* expression is considered crucial for proper AV-conduction and *Scn5a* heterozygous mice (mimicking a loss of function of the sodium channel) display AV-conduction dysfunction, including prolonged PR-interval and AV-block.[20] Therefore, the lower conduction reserve in the transitional zone of the AV-node due to low intrinsic *Scn5a* expression, in addition to a reduced peak I_{Na} due to the *Scn5a*$^{1798insD/+}$ mutation, may have contributed to the AV-conduction slowing in *Scn5a*$^{1798insD/+}$ mice. This observation may limit the transferability of our current findings to other (non-overlap) mutations. Nevertheless, the fact that chronic RAN treatment normalized AV-conduction demonstrates that enhanced $I_{Na,L}$ plays a major role. Moreover, the AV-conduction disturbances frequently observed in LQT3 patients suggests a more generally applicable mechanism relevant for gain-of-function *SCN5A* mutations.

Conclusion

This study identifies a functional role for chronically enhanced $I_{Na,L}$ in modulating AV-conduction via calcium-dependent (signalling) pathways in the setting of inherited sodium channelopathy. Our findings furthermore demonstrate a functional benefit of pharmacological

inhibition of $I_{Na,L}$ in abrogating AV-conduction disturbances secondary to gain-of-function *SCN5A* mutations.

Supplementary material

Supplementary material is available at *Europace* online.

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Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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