Inhibition of N-type Ca$^{2+}$ channels ameliorates an imbalance in cardiac autonomic nerve activity and prevents lethal arrhythmias in mice with heart failure

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Aims
Dysregulation of autonomic nervous system activity can trigger ventricular arrhythmias and sudden death in patients with heart failure. N-type Ca$^{2+}$ channels (NCCs) play an important role in sympathetic nervous system activation by regulating the calcium entry that triggers release of neurotransmitters from peripheral sympathetic nerve terminals. We have investigated the ability of NCC blockade to prevent lethal arrhythmias associated with heart failure.

Methods and results
We compared the effects of cilnidipine, a dual N- and L-type Ca$^{2+}$ channel blocker, with those of nitrendipine, a selective L-type Ca$^{2+}$ channel blocker, in transgenic mice expressing a cardiac-specific, dominant-negative form of neuron-restrictive silencer factor (dnNRSF-Tg). In this mouse model of dilated cardiomyopathy leading to sudden arrhythmic death, cardiac structure and function did not significantly differ among the control, cilnidipine, and nitrendipine groups. However, cilnidipine dramatically reduced arrhythmias in dnNRSF-Tg mice, significantly improving their survival rate and correcting the imbalance between cardiac sympathetic and parasympathetic nervous system activity. A β-blocker, bisoprolol, showed similar effects in these mice. Genetic titration of NCCs, achieved by crossing dnNRSF-Tg mice with mice lacking CACNA1B, which encodes the α1 subunit of NCCs, improved the survival rate. With restoration of cardiac autonomic balance, dnNRSF-Tg/CACNA1B$^{+/-}$ mice showed fewer malignant arrhythmias than dnNRSF-Tg/CACNA1B$^{-/-}$ mice.

Conclusions
Both pharmacological blockade of NCCs and their genetic titration improved cardiac autonomic balance and prevented lethal arrhythmias in a mouse model of dilated cardiomyopathy and sudden arrhythmic death. Our findings suggest that NCC blockade is a potentially useful approach to preventing sudden death in patients with heart failure.

Keywords
Ion channel • Nervous system • Autonomic • Heart failure • Arrhythmia • N-type Ca$^{2+}$ channel

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1. Introduction

Approximately 50% of deaths among patients with heart failure are classified as sudden death, mainly caused by lethal arrhythmias. Despite recent progress, pharmacological interventions for the treatment and prevention of lethal arrhythmias associated with chronic heart failure remain unsatisfactory. Nonetheless, it is anticipated that a better understanding of the molecular basis of arrhythmicity in failing hearts will enable identification of therapeutic targets that can serve as the basis for the development of new pharmaceutical treatments.

Autonomic dysregulation leading to increased sympathetic nerve activity and decreased parasympathetic nerve activity contributes to the increased arrhythmicity seen in patients with chronic heart failure. N-type voltage-dependent Ca\(^{2+}\) channels (NCCs), encoded by the CACNA1B (α1B subunit) gene, are predominantly localized in the nervous system, where they play a pivotal role in modulating a variety of neuronal functions, including neurotransmitter release at sympathetic nerve terminals. Mice lacking CACNA1B show functional deterioration of their sympathetic nervous system, and the ability of NCC blockade to prevent malignant arrhythmias and sudden death associated with heart failure remains unexplored.

We previously reported that transgenic mice cardiac-selectively expressing a dominant-negative form of neuron-restrictive silencer factor (NRSF, also called REST) (dnNRSF-Tg), a transcriptional repressor important for regulation of the fetal cardiac gene program, showed progressive cardiomyopathy and sudden arrhythmic death beginning at about 8 weeks of age. We have also reported several abnormalities in cardiac electrophysiological properties and ion channel expression in these dnNRSF-Tg mice. The dnNRSF-Tg hearts showed increased expression of fetal-type ion channel genes, including CACNA1H, which encodes the T-type Ca\(^{2+}\) channel (TCC) α1 subunit, and a corresponding increase in I\(_{Ca,T}\) amplitude. In that earlier study, we demonstrated that TCC blockade could prevent sudden death in dnNRSF-Tg mice by both restoring the normal electrophysiology of ventricular myocytes and correcting the cardiac autonomic dysfunction observed in dnNRSF-Tg mice. Because TCC expression, and thus functional TCC currents, is increased in the myocardium of dnNRSF-Tg mice, TCC blockade directly affects the electrophysiological properties of ventricular myocytes in dnNRSF-Tg mice. On the other hand, the impact of modulating autonomic nervous system balance on the incidence of lethal arrhythmias in dnNRSF-Tg mice remains unclear.

Pharmacological blockade or genetic deletion of NCCs reportedly alters autonomic activity in both human patients and animal models. On the other hand, little or no NCC expression has been detected in the ventricular myocardium. Therefore, to evaluate the extent to which correcting the autonomic imbalance prevents the lethal arrhythmias associated with heart failure, we assessed the effects of pharmacological blockade of NCCs and their genetic titration on arrhythmicity and sudden death in dnNRSF-Tg mice. Our findings demonstrate the importance of an imbalance between sympathetic and parasympathetic nerve activities in the generation of lethal arrhythmias in failing hearts and suggest that restoring autonomic nervous system balance through NCC inhibition can be an effective approach to preventing sudden arrhythmic death associated with heart failure.

2. Methods

An expanded Methods section is available in Supplementary material online.

2.1 Animal experiments

The animal care and all experimental protocols were reviewed and approved by the Animal Research Committee at Kyoto University Graduate School of Medicine, and conformed to the US National Institute of Health Guide for the Care and Use of Laboratory Animals. Beginning at 8 weeks of age, dnNRSF-Tg mice were left untreated (control) or were treated for 24 weeks with cilnidipine (10 mg/kg/day po) or nitrendipine (10 mg/kg/day po). The drug dosages were chosen based on earlier reports and our preliminary studies. Cilnidipine was supplied by Mochida Pharmaceutical Co., Ltd (Tokyo, Japan). Nitrendipine was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Bisoprolol was supplied by Mitsubishi Tanabe Pharma Corporation (Osaka, Japan). Cilnidipine exerts a much more potent inhibitory effect on N-type Ca\(^{2+}\) currents than does nitrendipine, which has little effect on N-type Ca\(^{2+}\) currents, particularly under conditions in which L-type Ca\(^{2+}\) current inhibition is comparable between the two drugs.

We then selected the doses of both drugs that similarly and minimally affected blood pressure. In another experiment, dnNRSF-Tg mice were bred with CACNA1B heterozygous knockout mice to obtain dnNRSF-Tg; CACNA1B\(^{+/-}\) mice and control dnNRSF-Tg; CACNA1B\(^{+/+}\) litters. CACNA1B\(^{+/-}\) mice were described in an earlier report. For the isolation and analysis of hearts, mice were anaesthetized with 3.0% of isoflurane and sacrificed by cervical dislocation.

2.2 Statistical analysis

Data are presented as means ± standard errors of the mean (SEM) unless indicated otherwise. Survival was analysed using the Kaplan–Meier method with the log-rank test. Comparisons among multiple groups were made using ANOVA with post hoc Fisher’s tests, except for numbers of arrhythmias. Values of P < 0.05 were considered significant. Numbers of arrhythmias between two groups were analysed using the Mann–Whitney test. Values of P < 0.05 were considered significant. Numbers of arrhythmias among four groups were analysed using Kruskal–Wallis non-parametric ANOVA followed by the Bonferroni correction. Values of P < 0.0083 were considered significant in that analysis.

3. Results

3.1 The dual N- and L-type Ca\(^{2+}\) channel blocker cilnidipine improves survival among dnNRSF-Tg mice without affecting cardiac structure or function

We initially confirmed that there is little expression of CACNA1B, encoding the α1 subunit of NCCs, in either wild-type (WT) or dnNRSF-Tg hearts, which is in contrast to its obvious expression in brain (Figure 1A). On the other hand, we detected substantially greater ventricular expression of CACNA1H, encoding the α1 subunit of TCCs, and CACNA1C, encoding the α1 subunit of L-type Ca\(^{2+}\) channels (Figure 1B). Although ventricular expression of CACNA1B is increased in dnNRSF-Tg hearts, probably due to the presence of NRSF-binding element in the gene, the levels are still lower than those of CACNA1H in WT hearts, where no functional T-type Ca\(^{2+}\) currents are detected. To evaluate the potential therapeutic effect of modulating autonomic nervous system activity through NCC blockade on the development of malignant arrhythmias and sudden death in dnNRSF-Tg mice, we administered suprpressor doses of cilnidipine, a dual N- and L-type dihydropyridine Ca\(^{2+}\) channel blocker, or nitrendipine, a more L-type-selective dihydropyridine Ca\(^{2+}\) channel blocker, to dnNRSF-Tg mice for 24 weeks, beginning when they were 8 weeks of age. Under our experimental conditions, systolic blood pressures and heart rates did not differ among the control, cilnidipine, and nitrendipine groups.
Pharmacological blockade of NCCs by cilnidipine improves survival among dnNRSF-Tg mice. (A) Relative levels of CACNA1B mRNA in brains (B) from WT, kidney (K) from WT, cardiac ventricle (V) from WT, and cardiac ventricle (V) from 8-week-old dnNRSF-Tg mice (Tg); levels in cardiac ventricle from WT mice were assigned a value of 1.0. \( n = 3 \) each for brain, kidney, and cardiac ventricle from WT mice and \( n = 2 \) for cardiac ventricle from dnNRSF-Tg mice. (B) Relative levels of CACNA1B, CACNA1H, and CACNA1C mRNA in cardiac ventricle from 8-week-old WT mice and dnNRSF-Tg mice (Tg); levels of CACNA1B mRNA in WT mice were assigned a value of 1.0. \( n = 5 \) for WT mice and \( n = 7 \) for dnNRSF-Tg. (C and D) Systolic blood pressures (C) and heart rates (D) in 20-week-old untreated WT, untreated Tg (Tg-cont), cilnidipine-treated Tg (Tg-Cil), and nitrendipine-treated Tg-Nit mice (\( n = 15 \) each for untreated Tg, Tg-Cil, and Tg-Nit, and \( n = 10 \) for untreated WT). ANOVA with post hoc Fisher’s tests was used for analysis. * \( P < 0.05 \), N.S.: not significant. (E) Kaplan–Meyer survival curves for untreated WT, untreated Tg, Cil-treated Tg, and Nit-treated Tg over a 24-week drug administration period (from 8 to 32 weeks of age): Log-rank test was used for analysis. * \( P < 0.05 \) \( n = 21 \) for WT, \( n = 23 \) for Tg without drugs, \( n = 22 \) for Tg + Cil, and \( n = 20 \) for Tg + Nit. The numbers of mice alive in each group at the end of each period are shown at the bottom of the figure. All data except survival curves are shown as means ± SEM.
of dnNRSF-Tg mice, though blood pressures were slightly lower and heart rates were significantly slower in dnNRSF-Tg mice than in untreated WT mice, as previously reported (systolic blood pressure: WT, 101.40 ± 1.48; Tg, 96.0 ± 1.75; Tg + cilnidipine, 96.67 ± 1.64; Tg + nitrendipine, 95.47 ± 1.92 mmHg and Heart rates: WT, 682.3 ± 27; dnNRSF-Tg, 590.6 ± 10.9; Tg + cilnidipine, 567.13 ± 17.58; Tg + nitrendipine, 568.8 ± 11.07/min) (Figure 1C and D). We found that cilnidipine dramatically improved the survival rate among dnNRSF-Tg mice, compared with mice treated with nitrendipine or untreated control (Figure 1E). Although heart-to-body weight ratios were higher in dnNRSF-Tg than in WT mice, as reported previously, heart-to-body weight ratios did not significantly differ among the control, cilnidipine, and nitrendipine groups of dnNRSF-Tg mice (WT, 4.08 ± 0.31; Tg, 5.94 ± 0.24; Tg + cilnidipine, 5.61 ± 0.48; Tg + nitrendipine, 5.94 ± 0.36 mg/g) (Figure 2A). Lung-to-body weight ratios also did not differ among these three groups (WT, 5.28 ± 0.37; Tg, 6.07 ± 0.22; Tg + cilnidipine, 5.93 ± 0.79; Tg + nitrendipine, 5.9 ± 0.29 mg/g) (Figure 2B). In addition, histological analyses, including determination of the %fibrotic area, and echocardiographic analyses also showed no significant differences among these three groups (Figure 2C–F and Table 1). In contrast, the echocardiography and histology showed that, compared with untreated WT mice, left ventricular systolic function was diminished and %fibrotic area was increased in dnNRSF-Tg mice, as reported previously (Figure 2C–F and Table 1). Consistent with these findings, there was no significant difference in the expression of two cardiac stress marker genes, ANP and SERCA2, among the three groups, whereas their expression did differ between untreated WT mice and dnNRSF-Tg mice, as described previously (Figure 2G and H).

Expression of the fibrosis-related genes Col1a1, Col3a1, and FN1, encoding collagen type1α1, collagen type3α1, and fibronectin 1, respectively, was not affected by the drug treatments (see Supplementary material online, Figure S1A–C). Expression of genes encoding the fetal-type ion channels CACNA1H, HCN2, and HCN4 was higher in untreated dnNRSF-Tg ventricles than in control WT ventricles, as reported previously, and cilnidipine did not affect expression of these genes in dnNRSF-Tg ventricles (see Supplementary material online, Figure S1D–F). Collectively, all of these data indicate that cilnidipine suppresses sudden death in dnNRSF-Tg mice without significantly affecting cardiac structure or function.

![Figure 2](image-url) Cilnidipine does not affect cardiac structure or function in dnNRSF-Tg mice. (A and B) Heart-to-body weight (HW/BW) ratios (A) and lung-to-body weight (LungW/BW) ratios (B) in 20-week-old untreated WT (WT-cont), untreated Tg (Tg-cont), Cil-treated Tg (Tg-Cil), and Nit-treated Tg (Tg-Nit) mice (n = 5 for untreated WT, n = 4 for Tg-cont, n = 4 for Tg-Cil, and n = 3 for Tg-Nit). (C) Histogram of hearts from 20-week-old untreated WT, Tg-cont, Tg-Cil, and Tg-Nit mice; H-E, haematoxylin-eosin staining; Sirius-red, Sirius-red staining. Scale bars = 100 μm. (D) %fibrotic area in 20-week-old untreated WT, Tg-cont, Tg-Cil, and Tg-Nit mice (n = 5 for untreated WT, n = 4 for Tg-cont, n = 7 for Tg-Cil, n = 7 for Tg-Nit). N.S.: not significant. (E and F) LVDd (E) and EF (F) assessed echocardiographically in untreated WT, Tg-cont, Tg-Cil, and Tg-Nit mice. *P < 0.05. N.S.: not significant. (n = 5 each for untreated WT, Tg-cont, Tg-Cil; n = 7 for Tg-Nit). (G and H) Relative levels of ANP (G) and SERCA2 (H) mRNA in cardiac ventricles from untreated WT, Tg-cont, Tg-Cil, and Tg-Nit mice; levels in untreated WT were assigned a value of 1.0. N.S.: not significant. (n = 5 each). ANOVA with post hoc Fisher’s tests was used for analysis. All data are shown as means ± SEM.
Table 1: Echocardiographic parameters in 20-week-old mice

<table>
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<tr>
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<th>WT Control</th>
<th>dnNRSF-Tg Cont</th>
<th>Cil</th>
<th>Nit</th>
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<td>LVDD (mm)</td>
<td>3.3 ± 0.13</td>
<td>3.9 ± 0.19</td>
<td>4.0 ± 0.11</td>
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<td>LVDS (mm)</td>
<td>2.1 ± 0.08</td>
<td>3.1 ± 0.17</td>
<td>3.1 ± 0.11</td>
<td>2.9 ± 0.10</td>
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<td>IVST (mm)</td>
<td>0.76 ± 0.02</td>
<td>0.72 ± 0.02</td>
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<td>PWT (mm)</td>
<td>0.76 ± 0.02</td>
<td>0.74 ± 0.02</td>
<td>0.76 ± 0.02</td>
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<tr>
<td>FS (%)</td>
<td>36.1 ± 2.3</td>
<td>20.3 ± 1.4</td>
<td>23.3 ± 2.7</td>
<td>23.8 ± 2.4</td>
</tr>
<tr>
<td>EF (%)</td>
<td>73.2 ± 2.7</td>
<td>49.0 ± 2.3</td>
<td>55.4 ± 4.2</td>
<td>57.0 ± 4.3</td>
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<table>
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<tr>
<th></th>
<th>WT 1B+/−</th>
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<th>Nit 1B+/−</th>
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<tr>
<td>LVDD (mm)</td>
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<td>3.3 ± 0.08</td>
<td>4.1 ± 0.12</td>
<td>3.3 ± 0.07</td>
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<td>LVDS (mm)</td>
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<td>IVST (mm)</td>
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<td>PWT (mm)</td>
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<tr>
<td>FS (%)</td>
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<tr>
<td>EF (%)</td>
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<td>68.9 ± 2.6</td>
<td>49.0 ± 2.4</td>
<td>64.3 ± 1.8</td>
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Values are means ± SEM. Cil, cilnidipine; Nit, nitrendipine; 1B+/−, CACNA1B+/−; 1B−/−, CACNA1B−/−; LVDD, left ventricular diastolic dimension; LVDS, left ventricular systolic dimension; FS, fractional shortening; IVST, intraventricular septum wall thickness; PWT, posterior wall thickness. Numbers of mice tested in the pharmacological inhibition study are as follows: n = 5 for WT, untreated dnNRSF-Tg, and Cil-treated dnNRSF-Tg; n = 7 for Nit-treated dnNRSF-Tg (upper panel). Numbers of mice tested in the genetic titration study are as follows: n = 13 for 1B+/−, n = 14 for 1B−/−, n = 11 for dnNRSF-Tg 1B+/−, and n = 15 for dnNRSF-Tg 1B−/− (lower panel). ANOVA with post hoc Fisher’s test was used for the analysis. *P < 0.05 vs. dnNRSF-Tg 1B+/−.

3.2 Cilnidipine improves cardiac autonomic nervous system function and reduces arrhythmicity in dnNRSF-Tg mice

We hypothesized that correcting autonomic balance through NCC blockade reduces arrhythmogenicity, thereby improving survival among dnNRSF-Tg mice. Heart rate variability (HRV) is a widely accepted index of cardiac autonomic nervous system activity. Earlier frequency domain analysis of HRV revealed that patients with severe heart failure show a progressive reduction in power in both the low-frequency (LF) and high-frequency (HF) ranges, and that a reduction in the LF power range is a significant predictor of sudden cardiac death in patients with heart failure. We used HRV as an index to evaluate cardiac autonomic function in WT and dnNRSF-Tg mice and examined the effects of cilnidipine on HRV. In mice, HRV predominantly correlates with parasympathetic activity. As we showed previously, both the LF and HF powers averaged over 24 h in dnNRSF-Tg mice (LF, 1.228 ± 0.198; HF, 0.823 ± 0.186 m/s²) were markedly lower than in WT mice (LF, 4.331 ± 0.706; HF, 2.412 ± 0.089 m/s²), indicating a general reduction in parasympathetic activity in dnNRSF-Tg mice (Figure 3A and B). Cilnidipine dramatically increased the power in both the LF and HF ranges of HRV (LF, 3.08 ± 0.338; HF, 2.228 ± 0.283 m/s²), whereas nitrendipine had little effect on HRV (LF, 0.538 ± 0.447; HF, 1.383 ± 0.57 m/s²) (Figure 3A and B). We also found that urinary excretion of norepinephrine, which is indicative of the level of sympathetic nervous system activity, was significantly higher in dnNRSF-Tg than in WT mice, and that norepinephrine excretion was significantly reduced only by cilnidipine (WT, 0.09 ± 0.02; Tg, 0.33 ± 0.04; Tg + cilnidipine, 0.15 ± 0.03; Tg + nitrendipine, 0.32 ± 0.1 µg/day) (Figure 3C).

We next used an implanted telemetric monitoring system to examine the effects of cilnidipine and nitrendipine on electrocardiographic parameters in dnNRSF-Tg mice. We found that only cilnidipine significantly suppressed the number of premature ventricular contractions (PVCs) in dnNRSF-Tg hearts (WT, 0 ± 0; dnNRSF-Tg, 502.66 ± 305.69; dnNRSF-Tg + cilnidipine, 1.0 ± 0.66; dnNRSF-Tg + nitrendipine, 326.17 ± 147.24/h) (Figure 3D). More importantly, it dramatically reduced the number of episodes of ventricular tachycardia (VT) (WT, 0 ± 0; dnNRSF-Tg, 14.92 ± 4.95; dnNRSF-Tg + cilnidipine, 0.06 ± 0.06; dnNRSF-Tg + nitrendipine, 12.75 ± 5.16/h) (Figure 3E and Supplementary material online, Figure S2A and B). These lines of evidence suggest that by restoring autonomic nervous system balance, cilnidipine reduces the incidence of lethal arrhythmias in dnNRSF-Tg mice.

3.3 β-Adrenergic receptor blockade prevents lethal arrhythmias and sudden death in dnNRSF-Tg mice

To verify the importance of correcting autonomic nervous system imbalance for the prevention of lethal arrhythmias and sudden death in dnNRSF-Tg mice, irrespective of effects on structural remodelling, we examined the effects of treating these mice with a β-adrenergic receptor blocker. We administered a subpressor dose of the lipophilic β-adrenergic receptor blocker bisoprolol (1 mg/kg/day po) to WT and dnNRSF-Tg mice. Although systolic blood pressures did not differ between untreated control and bisoprolol-treated mice (untreated WT, 107.5 ± 1.6; WT + bisoprolol, 108.0 ± 1.2; untreated Tg, 98.6 ± 2.0; Tg + bisoprolol, 98.6 ± 1.7 mmHg) (Figure 3F), heart rates were significantly slower in bisoprolol-treated than in untreated WT and dnNRSF-Tg mice (untreated WT, 697.8 ± 8.3; WT + bisoprolol,
604.7 ± 38.3; Tg, 601.6 ± 10.1; Tg + bisoprolol, 558.6 ± 12.0/min) (Figure 3G). At the dose tested, bisoprolol also did not affect cardiac systolic function assessed echocardiographically in dnNRSF-Tg mice [LVDD: WT, 3.3 ± 0.2; WT + bisoprolol, 3.2 ± 0.1; Tg, 3.9 ± 0.1; Tg + bisoprolol, 3.9 ± 0.1 mm and ejection fraction (EF): WT, 84.5 ± 4.0; WT + bisoprolol, 83.0 ± 1.5; Tg, 46.0 ± 1.6; Tg + bisoprolol, 51.5 ± 2.7%] (Figure 3H and I). On the other hand, bisoprolol significantly restored power in both the LF and HF ranges of HRV (LF: untreated WT, 5.19 ± 0.37; Tg, 1.36 ± 0.14; Tg + bisoprolol, 3.34 ± 0.39 m/s² and HF: untreated WT, 2.12 ± 0.24; Tg, 0.86 ± 0.12; Tg + bisoprolol, 1.62 ± 0.22 m/s² (Figure 3J and K) and reduced the incidence of PVCs and VTs in those mice (PVC: Tg, 408.3 ± 122.9; Tg + bisoprolol, 98.9 ± 42.2/h; VT: Tg, 28.2 ± 12.1; Tg + bisoprolol, 7.6 ± 1.7/h) (Figure 3L and M). As a result, bisoprolol significantly improved survival rates among dnNRSF-Tg mice (Figure 3N). These results strongly support our finding that imbalance of autonomic nervous system...
3.4 Genetic titration of NCC improves survival among dnNRSF-Tg mice

To further confirm the benefit of NCC inhibition for prevention of sudden death in dnNRSF-Tg mice, we next genetically titrated NCC expression by crossing dnNRSF-Tg mice with mice lacking CACNA1B, encoding the α1B subunit of NCC. Because the CACNA1B−/− genotype has a high incidence of early mortality from an as yet unknown cause, we compared the phenotypes of dnNRSF-TgCACNA1B+/− mice with those of dnNRSF-TgCACNA1B+/− mice, in which NCC expression is reduced to ~52.9% of that in dnNRSF-TgCACNA1B+/− mice (Figure 4A). The gross appearance of CACNA1B+/− mice is normal, and they show no early mortality. Systolic blood pressures in dnNRSF-TgCACNA1B+/− and dnNRSF-TgCACNA1B+/− mice did not significantly differ, but they were mildly lower than in control WT (CACNA1B+/−) mice (WT, 101.25 ± 7.26; CACNA1B+/−, 91.25 ± 2.78; dnNRSF-Tg, 92 ± 4.38; dnNRSF-TgCACNA1B+/−, 89.25 ± 2.14 mmHg) (Figure 4B). Similarly, heart rates did not differ between dnNRSF-TgCACNA1B+/− and dnNRSF-TgCACNA1B+/− mice, although they were slower in dnNRSF-TgCACNA1B+/− than in control WT mice, as reported previously (WT, 632.25 ± 26.36; CACNA1B+/−, 594 ± 33.39; dnNRSF-Tg, 515.25 ± 14.71; dnNRSF-TgCACNA1B+/−, 521.5 ± 23.32/min) (Figure 4C).8 Body weights were comparable between the two dnNRSF-Tg groups (WT, 31.08 ± 1.11; CACNA1B+/−, 29.53 ± 1.37; dnNRSF-Tg, 28.86 ± 1.19; dnNRSF-TgCACNA1B+/−, 27.41 ± 1.09 g) (Figure 4D), but heart-to-body weight ratios were higher in dnNRSF-TgCACNA1B+/− than in WT (CACNA1B+/−) mice and were significantly lower in dnNRSF-TgCACNA1B+/− than in dnNRSF-TgCACNA1B+/− mice (WT, 4.44 ± 0.04; CACNA1B+/−, 4.51 ± 0.14; dnNRSF-Tg, 5.68 ± 0.21; dnNRSF-TgCACNA1B+/−, 4.86 ± 0.18 mg/g) (Figure 4E). Lung-to-body weight ratios were comparable between the two dnNRSF-Tg groups (WT, 5.06 ± 0.22; CACNA1B+/−, 4.68 ± 0.96; dnNRSF-Tg, 5.41 ± 0.09; dnNRSF-TgCACNA1B+/−, 5.52 ± 0.26 mg/g) (Figure 4F). Echocardiographic analysis showed that left ventricular diastolic dimension (LVDd) was higher in dnNRSF-TgCACNA1B+/− than in WT mice, whereas EF was lower in dnNRSF-TgCACNA1B+/− than in WT mice, as was reported previously (Figure 5A and B).8 In addition, LVDd was lower and EF was higher in dnNRSF-TgCACNA1B+/− than in dnNRSF-TgCACNA1B+/− mice (Figure 5A and B and Table 1). Histological analysis revealed no significant difference between dnNRSF-TgCACNA1B+/− and dnNRSF-TgCACNA1B+/− mice, although %fibrotic area showed a trend towards being smaller in dnNRSF-TgCACNA1B+/− than in dnNRSF-TgCACNA1B+/− mice (Figure 5C and D). Expression of the fibrosis-related genes Col1α1, Col3α1, and FN1 did not significantly differ between dnNRSF-TgCACNA1B+/− and dnNRSF-TgCACNA1B+/− mice (see Supplementary material online, Figure S3A–C). Col3α1, and FN1 did not significantly differ between dnNRSF-TgCACNA1B+/− and dnNRSF-TgCACNA1B+/− mice (see Supplementary material online, Figure S3A–C). Col3α1, and FN1 did not significantly differ between dnNRSF-TgCACNA1B+/− and dnNRSF-TgCACNA1B+/− mice (see Supplementary material online, Figure S3A–C). Col3α1, and FN1 did not significantly differ between dnNRSF-TgCACNA1B+/− and dnNRSF-TgCACNA1B+/− mice (see Supplementary material online, Figure S3A–C).

3.5 Reducing CACNA1B expression improves autonomic function and decreases the occurrence of arrhythmias in dnNRSF-Tg mice

We also assessed autonomic nervous system activity in dnNRSF-TgCACNA1B+/− and dnNRSF-TgCACNA1B+/− mice. In HRV analyses, the reductions in LF and HF power otherwise seen in dnNRSF-TgCACNA1B+/− mice (LF, 1.288 ± 0.16; HF, 1.168 ± 0.108 m/s²) were significantly ameliorated in dnNRSF-TgCACNA1B+/− mice (LF, 3.54 ± 0.47; HF, 3.075 ± 0.468 m/s²), indicating a restoration of parasympathetic activity through reduction of NCC function (Figure 6B and C). In addition, we found that the increase in urinary excretion of norepinephrine seen in dnNRSF-TgCACNA1B+/− mice (0.428 ± 0.07 µg/day) was significantly ameliorated in dnNRSF-TgCACNA1B+/− mice (0.154 ± 0.05 µg/day) (Figure 6D). Finally, evaluation of arrhythmia revealed that the incidences of both PVCs and VT were significantly lower in dnNRSF-TgCACNA1B+/− than in dnNRSF-TgCACNA1B+/− mice (PVC: WT, 0 ± 0; CACNA1B+/−, 0 ± 0); dnNRSF-Tg, 239.08 ± 27.93; dnNRSF-TgCACNA1B+/−, 3.21 ± 3.21 and VT: WT, 0 ± 0; CACNA1B+/−, 0 ± 0; dnNRSF-Tg, 41.3 ± 12.69; dnNRSF-TgCACNA1B+/−, 0.36 ± 0.36/h) (Figure 6E and F). These results demonstrate that genetic titration of CACNA1B, encoding NCC, corrected an imbalance between sympathetic and parasympathetic nervous system activities, which, at least in part, contributed to reducing malignant arrhythmias in dnNRSF-Tg mice in a manner similar to pharmacological NCC blockade.

4. Discussion

Autonomic dysregulation leading to increased sympathetic nerve activity and reduced parasympathetic nerve activity is reportedly associated with the increased arrhythmia seen in patients with chronic heart failure. NCCs may play a major role in the release of norepinephrine at sympathetic nerve terminals. Consequently, mice lacking CACNA1B, the gene encoding the α1B subunit of NCCs, exhibit a significantly impaired positive inotropic response. In the present study, we found that pharmacological blockade of NCCs or their genetic titration improved the balance between sympathetic and parasympathetic nerve activities and prevented the sudden death and arrhythmicity otherwise seen in dnNRSF-Tg mice, a mouse model of sudden arrhythmic death associated with cardiac dysfunction. The mode of death in these model mice is sudden and without overt oedema, pleural effusion, or apparent lung congestion, and all the telemetry data obtained at the time of death indicate VT/VF to be the cause. Moreover, in an earlier study, we found that systemic administration of isoproterenol induced VT more frequently in dnNRSF-Tg than in WT mice. Conversely, administration of a β-blocker led to a significant reduction in the incidence of sudden death among dnNRSF-Tg mice under conditions in which cardiac systolic function and remodelling were not affected (Figure 3H–N). These findings suggest that NCC blockade or genetic titration of NCC reduces the likelihood of sudden arrhythmic death, thereby improving survival.

Pharmacological interventions that reduce cardiac sympathetic activity have been shown to protect against arrhythmias, while...
interventions that stimulate cardiac sympathetic activity provoke malignant arrhythmias. In patients with heart failure, β-adrenergic receptor blockade reduces the incidence of sudden death; however, β-blockers are not completely protective, and mortality remains high among patients with cardiac dysfunction, despite optimal β-blocker therapy. It is therefore necessary to find other approaches to modulate sympathetic or parasympathetic activity. In that context, a clinical trial testing the effect of central modulation of sympathetic activity using moxonidine SR in patients with heart failure was terminated early due to an increase in mortality and morbidity in patients receiving the drug. Thus, strong central inhibition of the sympathetic nervous system through imidazoline receptor stimulation appears not to

Figure 4 Effects of genetic titration of CACNA1B on hemodynamics and heart size in WT and dnNRSF-Tg mice. (A) CACNA1B mRNA expression in brains from 8-week-old CACNA1B+/+, CACNA1B+/−, and CACNA1B−/− mice; the level in CACNA1B+/+ brain was assigned a value of 1.0. (B and C) Systolic blood pressures (B) and heart rates (C) in 20-week-old CACNA1B+/+, CACNA1B+/−, dnNRSF-TgCACNA1B+/+, and dnNRSF-TgCACNA1B+/− mice. N.S.: not significant (n = 4 each). (D, E, and F) Body weights (BW) (D), heart-to-body weight ratios (HW/BW) (E), and lung-to-body weight ratios (Lung/BW) (F) in 20-week-old CACNA1B+/+, CACNA1B+/−, dnNRSF-TgCACNA1B+/+, and dnNRSF-TgCACNA1B+/− mice. *P < 0.05. N.S.: not significant. (BW and HW/BW: n = 4 for CACNA1B+/+, n = 6 for CACNA1B+/−, n = 5 for dnNRSF-TgCACNA1B+/+, and n = 7 for dnNRSF-TgCACNA1B+/−; Lung/W/BW: n = 4 for CACNA1B+/+, n = 6 for CACNA1B+/− and dnNRSF-TgCACNA1B+/−, and n = 5 for dnNRSF-TgCACNA1B+/+). ANOVA with post hoc Fisher’s tests was used for analysis. All data are shown as means ± SEM.
ability to block L-type Ca2+ channels has a detrimental effect on cardiac function, though it did prevent lethal arrhythmias, or that the NCC inhibition achieved in 20-week-old CACNA1B+/+/dnNRSF-Tg;CACNA1B+/− mice was more prolonged and more stable than that achieved with cilnidipine, which was not started until the mice were 8 weeks of age. The effects of genetic titration of CACNA1B+/+, CACNA1B+/−, dnNRSF-Tg;CACNA1B+/+ and dnNRSF-Tg;CACNA1B+/− mice. H-E, haematoxylin-eosin staining; Sirius-red, Sirius-red staining. Scale bars = 100 μm. (D) %Fibrotic area in the indicated groups (n = 4 for CACNA1B+/+, n = 6 for CACNA1B+/−, n = 5 for dnNRSF-Tg;CACNA1B+/+, and n = 7 for dnNRSF-Tg;CACNA1B+/−). N.S.: not significant. (E and F) Relative levels of ANP and SERCA2 mRNA in cardiac ventricles from 20-week-old CACNA1B+/+, CACNA1B+/−, dnNRSF-Tg;CACNA1B+/+ and dnNRSF-Tg;CACNA1B+/− mice (n = 4 for CACNA1B+/+, n = 6 for CACNA1B+/−, and n = 5 for dnNRSF-Tg;CACNA1B+/+, and dnNRSF-Tg;CACNA1B+/−); levels in CACNA1B+/+ ventricles were assigned a value of 1.0. *P < 0.05. ANOVA with post hoc Fisher’s tests was used for analysis. All data are shown as means ± SEM.

Figure 5 Effect of genetic titration of CACNA1B on cardiac structure and function in dnNRSF-Tg mice. (A and B) LVd and EF assessed echocardiographically in 20-week-old CACNA1B+/+, CACNA1B+/−, CACNA1B+/−; dnNRSF-Tg and CACNA1B+/−; dnNRSF-Tg mice.*P < 0.05, (n = 13 for CACNA1B+/+, n = 14 for CACNA1B+/−, n = 11 for dnNRSF-TgCACNA1B+/+; and n = 15 for dnNRSF-TgCACNA1B+/−). (C) Histology of hearts from 20-week-old CACNA1B+/+, CACNA1B+/−, dnNRSF-TgCACNA1B+/+ and dnNRSF-Tg;CACNA1B+/− mice. H-E, haematoxylin-eosin staining; Sirius-red, Sirius-red staining. Scale bars = 100 μm. (D) %Fibrotic area in the indicated groups (n = 4 for CACNA1B+/+, n = 6 for CACNA1B+/−, n = 5 for dnNRSF-Tg;CACNA1B+/+, and n = 7 for dnNRSF-Tg;CACNA1B+/−). N.S.: not significant. (E and F) Relative levels of ANP and SERCA2 mRNA in cardiac ventricles from 20-week-old CACNA1B+/+, CACNA1B+/−, dnNRSF-Tg;CACNA1B+/+ and dnNRSF-Tg;CACNA1B+/− mice (n = 4 for CACNA1B+/+, n = 6 for CACNA1B+/−, and n = 5 for dnNRSF-Tg;CACNA1B+/+, and dnNRSF-Tg;CACNA1B+/−); levels in CACNA1B+/+ ventricles were assigned a value of 1.0. *P < 0.05. ANOVA with post hoc Fisher’s tests was used for analysis. All data are shown as means ± SEM.

protect against lethal arrhythmias. NCCs are localized at peripheral sympathetic nerve terminals, where they regulate the release of neurotransmitters (e.g. catecholamines), thereby modulating sympathetic activity.4–6 Our findings suggest that, by correcting their autonomic dysregulation, NCC blockade could be an effective approach to preventing sudden arrhythmic death in patients with heart failure.

Cilnidipine failed to prevent the decline in cardiac function in dnNRSF-Tg mice, whereas genetic titration tended to ameliorate the adverse cardiac remodelling and cardiac dysfunction seen in dnNRSF-Tg mice (Figures 2A–H, 4E, and 5A–F and Table 1). The reasons for the difference in the effects on cardiac function between cilnidipine and genetic titration of NCCs remain unclear at present. It may be that cilnidipine’s ability to block L-type Ca2+ channels has a detrimental effect on cardiac function, as L-type Ca2+ channel blockers can adversely affect the progression of heart failure.10 Other possibilities are that the relatively low dose of cilnidipine used in this study was not sufficient to prevent the progression of cardiac dysfunction, though it did prevent lethal arrhythmias, or that the NCC inhibition achieved in CACNA1B+/− mice was more prolonged and more stable than that achieved with cilnidipine, which was not started until the mice were 8 weeks of age. The effects on NCCs expressed in the central nervous system could also differ between cilnidipine and genetic titration, as cilnidipine has little ability to cross the blood–brain barrier.11 These differences suggest the underlying mechanisms involved in the reduced incidence of lethal arrhythmias, and the prolonged survival differ somewhat between cilnidipine treatment and genetic titration of CACNA1B in this study. Cilnidipine treatment, which improved autonomic imbalance and reduced lethal arrhythmias without affecting cardiac remodelling, mainly suppressed the triggering of lethal arrhythmias induced by autonomic imbalance. On the other hand, genetic titration of CACNA1B, which improved autonomic imbalance and also tended to prevent adverse cardiac remodelling, suppressed lethal arrhythmias and improved survival in two ways: it inhibited the triggering of arrhythmias and also suppressed the generation of arrhythmogenic substrates. In both cases, correcting the autonomic imbalance associates with a reduction in the incidence of sudden death attributable to lethal arrhythmias in dnNRSF-Tg mice. However, because it is not possible to completely exclude the possibility that some dnNRSF-Tg mice (especially older mice) die due to congestive heart failure, irrespective of arrhythmias, there is a possibility that genetic deletion of NCC may also prevent this mode of death in addition to sudden arrhythmic death in dnNRSF-Tg mice through suppression of excessive sympathetic activity.

In the present study, both pharmacological blockade of NCCs and their genetic titration not only repressed sympathetic activity, as demonstrated by a reduction in urinary norepinephrine levels, but also restored parasympathetic activity, as indicated by HRV analyses. The precise
Although further investigation is necessary, our study contributes to the protective effects of NCC inhibition observed in sympathetic activity, an increase in parasympathetic activity likely results from the balance of the neuraxis. This suggests that, in addition to a reduction in sympathetic activity, ameliorating the reduction in parasympathetic activity, as found in hypertensive patients while exerting a concomitant sympathoinhibitory effect. Moreover, there is now much evidence showing the anti-arrhythmic effects of parasympathetic nervous activation. That is, cilnidipine reportedly enhances parasympathetic activity in hypertensive patients while exerting a concomitant sympathoinhibitory effect. Moreover, there is now much evidence showing the anti-arrhythmic effects of parasympathetic nervous activation. That is, cilnidipine reportedly enhances parasympathetic activity in hypertensive patients while exerting a concomitant sympathoinhibitory effect. Moreover, there is now much evidence showing the anti-arrhythmic effects of parasympathetic nervous activation. That is, cilnidipine reportedly enhances parasympathetic activity in hypertensive patients while exerting a concomitant sympathoinhibitory effect. Moreover, there is now much evidence showing the anti-arrhythmic effects of parasympathetic nervous activation. That is, cilnidipine reportedly enhances parasympathetic activity in hypertensive patients while exerting a concomitant sympathoinhibitory effect. Moreover, there is now much evidence showing the anti-arrhythmic effects of parasympathetic nervous activation. That is, cilnidipine reportedly enhances parasympathetic activity in hypertensive patients while exerting a concomitant sympathoinhibitory effect. Moreover, there is now much evidence showing the anti-arrhythmic effects of parasympathetic nervous activation. That is, cilnidipine reportedly enhances parasympathetic activity in hypertensive patients while exerting a concomitant sympathoinhibitory effect. Moreover, there is now much evidence showing the anti-arrhythmic effects of parasympathetic nervous activation. That is, cilnidipine reportedly enhances parasympathetic activity in hypertensive patients while exerting a concomitant sympathoinhibitory effect. Moreover, there is now much evidence showing the anti-arrhythmic effects of parasympathetic nervous activation. That is, cilnidipine reportedly enhances parasympathetic activity in hypertensive patients while exerting a concomitant sympathoinhibitory effect. Moreover, there is now much evidence showing the anti-arrhythmic effects of parasympathetic nervous activation. That is, cilnidipine reportedly enhances parasympathetic activity in hypertensive patients while exerting a concomitant sympathoinhibitory effect. Moreover, there is now much evidence showing the anti-arrhythmic effects of parasympathetic nervous activation. That is, cilnidipine reportedly enhances parasympathetic activity in hypertensive patients while exerting a concomitant sympathoinhibitory effect. Moreover, there is now much evidence showing the anti-arrhythmic effects of parasympathetic nervous activation. That is, cilnidipine reportedly enhances parasympathetic activity in hypertensive patients while exerting a concomitant sympathoinhibitory effect. Moreover, there is now much evidence showing the anti-arrhythmic effects of parasympathetic nervous activation. That is, cilnidipine reportedly enhances parasympathetic activity in hypertensive patients while exerting a concomitant sympathoinhibitory effect. Moreover, there is now much evidence showing the anti-arrhythmic effects of parasympathetic nervous activation. That is, cilnidipine reportedly enhances parasympathetic activity in hypertensive patients while exerting a concomitant sympathoinhibitory effect.

**Figure 6** Genetic titration of CACNA1B restores cardiac autonomic nervous system balance and reduces arrhythmias in dnNRSF-Tg mice. (A) Kaplan–Meier survival curves for CACNA1B+/+ (1B+/+), CACNA1B−/− (1B−/−), dnNRSF-TgCACNA1B+/+ (Tg1B+/+), and dnNRSF-TgCACNA1B−/− (Tg1B−/−) mice. Curves cover the period from birth to 32 weeks of age. Log-rank test was used for analysis. *P < 0.05 (n = 22 for CACNA1B+/+, n = 38 for CACNA1B−/−, n = 20 for dnNRSF-TgCACNA1B+/+, and n = 42 for dnNRSF-TgCACNA1B−/−). The numbers of mice alive in each group at the end of each period are shown at the bottom of the figure. (B and C) Average power of the LF (B) and HF (C) components of heart rate variability (HRV) recorded over a 24-h period in 20-week-old CACNA1B+/+, CACNA1B−/−, dnNRSF-TgCACNA1B+/+, and dnNRSF-TgCACNA1B−/− mice. *P < 0.05 (n = 5 for CACNA1B+/+, n = 7 for CACNA1B−/−, n = 6 for dnNRSF-TgCACNA1B+/+, and n = 7 for dnNRSF-TgCACNA1B−/−). (D) Urinary norepinephrine (NE) levels in 20-week-old CACNA1B+/+, CACNA1B−/−, dnNRSF-TgCACNA1B+/+, and dnNRSF-TgCACNA1B−/− mice. *P < 0.05 (n = 5 for CACNA1B+/+, n = 6 for CACNA1B−/−, n = 6 for dnNRSF-TgCACNA1B+/+, and n = 7 for dnNRSF-TgCACNA1B−/−). (E and F) Numbers of PVC (E) and VT (F) recorded using a telemetry system in 20-week-old CACNA1B+/+, CACNA1B−/−, dnNRSF-TgCACNA1B+/+, and dnNRSF-TgCACNA1B−/− mice are shown by dot plot. *P < 0.0083 (n = 5 for CACNA1B+/+, n = 7 for CACNA1B−/−, n = 6 for dnNRSF-TgCACNA1B+/+, and n = 7 for dnNRSF-TgCACNA1B−/−). All data in B–D are shown as means ± SEM. ANOVA with post hoc Fisher’s tests was used for analysis, except for numbers of arrhythmias (E and F). Numbers of arrhythmias were analyzed using Kruskal–Wallis non-parametric ANOVA followed by the Bonferroni correction.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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