Heart Failure

T-Type Ca\textsuperscript{2+} Channel Blockade Prevents Sudden Death in Mice With Heart Failure

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Background—Pharmacological interventions for prevention of sudden arrhythmic death in patients with chronic heart failure remain limited. Accumulating evidence suggests increased ventricular expression of T-type Ca\textsuperscript{2+} channels contributes to the progression of heart failure. The ability of T-type Ca\textsuperscript{2+} channel blockade to prevent lethal arrhythmias associated with heart failure has never been tested, however.

Methods and Results—We compared the effects of efonidipine and mibefradil, dual T- and L-type Ca\textsuperscript{2+} channel blockers, with those of nitrendipine, a selective L-type Ca\textsuperscript{2+} channel blocker, on survival and arrhythmogenicity in a cardiac-specific, dominant-negative form of neuron-restrictive silencer factor transgenic mice (dnNRSF-Tg), which is a useful mouse model of dilated cardiomyopathy leading to sudden death. Efonidipine, but not nitrendipine, substantially improved survival among dnNRSF-Tg mice. Arrhythmogenicity was dramatically reduced in dnNRSF-Tg mice treated with efonidipine or mibefradil. Efonidipine acted by reversing depolarization of the resting membrane potential otherwise seen in ventricular myocytes from dnNRSF-Tg mice and by correcting cardiac autonomic nervous system imbalance. Moreover, the R(−)-isomer of efonidipine, a recently identified, highly selective T-type Ca\textsuperscript{2+} channel blocker, similarly improved survival among dnNRSF-Tg mice. Efonidipine also reduced the incidence of sudden death and arrhythmogenicity in mice with acute myocardial infarction.

Conclusions—T-type Ca\textsuperscript{2+} channel blockade reduced arrhythmias in a mouse model of dilated cardiomyopathy by repolarizing the resting membrane potential and improving cardiac autonomic nervous system imbalance. T-type Ca\textsuperscript{2+} channel blockade also prevented sudden death in mice with myocardial infarction. Our findings suggest T-type Ca\textsuperscript{2+} channel blockade is a potentially useful approach to preventing sudden death in patients with heart failure. (Circulation. 2009;120:743-752.)

Key Words: ion channels ■ nervous system, autonomic ■ heart failure ■ calcium ■ arrhythmia

As many as 50% of deaths among heart failure patients are sudden and unexpected, presumably the result of lethal arrhythmias.\textsuperscript{1} Despite recent progress in nonpharmacological therapy, pharmacological interventions for the treatment and prevention of lethal arrhythmias associated with chronic heart failure remain limited. A prerequisite for the development of new pharmacological approaches is to identify potential targets based on knowledge of the molecular basis of arrhythmogenesis in failing hearts.

Clinical Perspective on p 752

Compelling evidence implicates T-type Ca\textsuperscript{2+} channels in the progression of heart failure.\textsuperscript{2,3} During development, T-type Ca\textsuperscript{2+} channels are abundantly expressed in the embryonic ventricle, but their expression is suppressed in the adult ventricle, so that it is restricted to the conduction system.\textsuperscript{4,5} However, T-type Ca\textsuperscript{2+} channels are reexpressed in hypertrophied and failing ventricles,\textsuperscript{6,6–9} and the resultant T-type Ca\textsuperscript{2+} currents (I_{Ca,T}) are thought to be involved in the pathological process that leads to systolic dysfunction and arrhythmogenesis.\textsuperscript{2,9} Indeed, several studies have shown that mibefradil, which blocks both T- and L-type Ca\textsuperscript{2+} channels, mitigates the functional deterioration of the ventricle in some animal models of heart failure.\textsuperscript{10–12} More recently, it was shown that the genetic deletion of CACNA1H, which encodes the α1H T-type Ca\textsuperscript{2+} channel, resulted in resistance to pathological...
cardiac hypertrophy. The ability of T-type Ca\(^{2+}\) channel blockade to prevent malignant arrhythmia and sudden death associated with heart failure remains unexplored, however.

We recently reported that a transcriptional repressor, neuron-restrictive silencer factor (NRSF, also called REST), is an important regulator of the fetal cardiac gene program.\(^{13}\) Transgenic mice that selectively express a dominant-negative form of NRSF (dnNRSF) in their hearts (dnNRSF-Tg) showed progressive cardiomyopathy and sudden arrhythmic death beginning at \(~8\) weeks of age.\(^{14}\) The dnNRSF-Tg hearts showed increased expression of fetal-type ion channel genes, including \(CACNA1H\), which encodes the T-type Ca\(^{2+}\) channel \(\alpha\)-subunit. Moreover, \(I_{\text{Ca,T}}\) amplitude was correspondingly increased in ventricular myocytes from dnNRSF-Tg hearts, which suggests that \(I_{\text{Ca,T}}\) in some way contributes to the susceptibility of dnNRSF-Tg hearts to arrhythmias.\(^{14}\)

To clarify the contribution made by T-type Ca\(^{2+}\) channels to the development of malignant arrhythmias and to assess the ability of T-type Ca\(^{2+}\) channel blockade to prevent sudden death associated with heart failure, we compared the effects of efonidipine and mibefradil, dual T- and L-type Ca\(^{2+}\) channel blockers,\(^{15,16}\) with those of nitrendipine, a more L-type-selective Ca\(^{2+}\) channel blocker, on survival and arrhythmogenicity in dnNRSF-Tg mice and mice with myocardial infarction. We also tested the effects of the \(R\) (-)-isomer of efonidipine \([R\) (-)-efonidipine], a recently identified, highly selective T-type Ca\(^{2+}\) channel blocker, on dnNRSF-Tg mice.\(^{17,18}\) Our findings demonstrate that T-type Ca\(^{2+}\) channel blockade may represent a new and effective means of preventing sudden cardiac death in patients with heart failure.

Methods

Animal Experiments

Animal care and all experimental protocols were conducted in accordance with the institutional guidelines of the Kyoto University Graduate School of Medicine. Beginning at 8 weeks of age, dnNRSF-Tg mice were left untreated (control) or were treated for 7 weeks with efonidipine (40 mg \(\cdot\) kg\(^{-1}\) \(\cdot d\)\(^{-1}\) PO) or nitrendipine (20 mg \(\cdot\) kg\(^{-1}\) \(\cdot d\)\(^{-1}\) PO). In another experiment, 10- or 11-week-old dnNRSF-Tg mice were left untreated (control) or treated for 7 days with mibefradil (15 mg \(\cdot\) kg\(^{-1}\) \(\cdot d\)\(^{-1}\)), efonidipine (200 mg \(\cdot\) kg\(^{-1}\) \(\cdot d\)\(^{-1}\)), or nitrendipine (60 mg \(\cdot\) kg\(^{-1}\) \(\cdot d\)\(^{-1}\)). The doses of mibefradil, efonidipine, and nitrendipine were chosen on the basis of earlier reports and our preliminary studies.\(^{19-22}\)

In the experiment with \(R\) (-)-efonidipine, dnNRSF-Tg mice were left untreated (control) or were treated for 20 weeks with the \(R\) (-)-isomer efonidipine (200 mg \(\cdot\) kg\(^{-1}\) \(\cdot d\)\(^{-1}\) PO). Acute myocardial infarction was induced in female C57BL/6 mice (age 8 to 12 weeks; weight 19 to 24 g) by ligation of the left coronary artery as described previously.\(^{23}\) Beginning 1 day after the operation, mice were left untreated (control) or were treated for 30 days with efonidipine hydrochloride (200 mg \(\cdot\) kg\(^{-1}\) \(\cdot d\)\(^{-1}\)) or nitrendipine (60 mg \(\cdot\) kg\(^{-1}\) \(\cdot d\)\(^{-1}\)).

Efonidipine and \(R\) (-)-isomer efonidipine were supplied by Nissan Chemical Industries, Ltd (Tokyo, Japan). Nitrendipine was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan).

Patch-Clamp Studies

Myocytes were dispersed by a method described previously.\(^{24}\) To record T- and L-type Ca\(^{2+}\) currents, electrodes were filled with Cs\(^{+}\)-rich solution that contained (in mmol/L): 100 CsCl, 50 NMDG, 10 TEA, 5 MgATP, 5 HEPES, and 10 EGTA (pH 7.2 with CsOH). After establishment of the ruptured whole-cell patch configuration in normal Tyrode solution, the bathing solution was switched to Na\(^{+}\)-free solution. A stock solution of efonidipine (Efo; 10 \(\mu\)mol/L) on \(I_{\text{Ca,T}}\) (T) and \(I_{\text{Ca,L}}\) (L). In the top panel, \(I_{\text{Ca,T}}\) was activated by depolarization to \(-45\) mV from a holding potential of \(-80\) mV. Bottom panel, \(I_{\text{Ca,L}}\) recorded from a myocyte from a WT mouse. \(I_{\text{Ca,L}}\) was activated by depolarization to \(-10\) mV from a holding potential of \(-80\) mV. E, Summary of the inhibitory effects of efonidipine (Efo; 10 \(\mu\)mol/L on \(I_{\text{Ca,T}}\) (T) and \(I_{\text{Ca,L}}\) (L) in ventricular myocytes. Efonidipine reduced the amplitudes of \(I_{\text{Ca,T}}\) by \(57\pm12\%\) (n=4) while reducing the amplitudes of \(I_{\text{Ca,L}}\) by \(21\pm4\%\) (n=5).

Intracardiac Electrophysiology

A 1.7F octapolar catheter (CIBer mouse EP, NuMe, Hopkinton, NY) inserted via the jugular vein was used to perform a standard electrophysiological study protocol as described previously.\(^{14,25}\)

Statistical Analysis

Data are presented as mean\(\pm\)SEM. Survival was analyzed by the Kaplan-Meier method with the log-rank test. ANOVA with post hoc Student-Newman-Keuls tests were used for comparisons among groups. Values of \(P<0.05\) were considered significant. Repeated-measures analyses with linear mixed-effects models were performed with data comprising repeated observations made over time. Data obtained from the 2-way factorial design were analyzed with the 2-way ANOVA.
hearts (Figure 1A). To determine the role played by T-type Ca\textsuperscript{2+} in adult ventricular myocytes from wild-type littermate (WT) (Figure 1A through 1C). By contrast, no blocker, to dnNRSF-Tg mice for 7 weeks, beginning when they were 8 weeks of age. Initially, we confirmed that efonidipine significantly blocked heart weight–to–body weight (HW/BW) ratios (B) and lung weight–to–body weight (LW/BW) ratios (C) in 12-week-old WT and Tg mice, with or without Efo or Nit. *P<0.05 vs WT (n=10 for each group). D and E, Left ventricular diastolic dimension (LVDd; D) and % fractional shortening (FS; E), assessed echocardiographically in WT and Tg mice, with or without Efo or Nit, during a 4-week period beginning when the mice were 8 weeks of age (n=4 for WT, n=5 for Tg without drugs, n=6 for Tg with Efo, n=5 for Tg with Nit). The comparison of trends in LVDd and FS over time among Tg, Tg with Efo, and Tg with Nit, by repeated-measures analyses with linear mixed-effects models, showed no statistical significance (LVDd, P=0.689; FS, P=0.735). F, Histology of WT and dnNRSF-Tg hearts from 12-week-old mice treated with or without Efo or Nit. Hematoxylin-and-eosin staining; magnification ×400. Scale bars=20 μm. G–J, Relative levels of BNP (G), SERCA2 (H), CACNA1H (I), and CACNA1G (J) mRNA expression in hearts from 12-week-old WT and Tg mice treated with or without Efo or Nit. *P<0.05 vs WT, †P<0.05 vs Tg without drugs (n=4 for WT, n=5 for Tg without drugs, n=4 for Tg with Efo, n=4 for Tg with Nit).

**Results**

**Dual T- and L-Type Ca\textsuperscript{2+} Channel Blocker Efonidipine Improves Survival Among dnNRSF-Tg Mice**

We previously showed that dnNRSF-Tg mice develop progressive cardiomyopathy and begin to die of ventricular tachyarrhythmias at ~8 weeks of age. In dnNRSF-Tg hearts, CACNA1H, the gene that encodes the T-type Ca\textsuperscript{2+} channel α-subunit and a transcriptional target of NRSF/REST, was upregulated, and there was a corresponding increase in $I_{Ca,T}$ amplitude in the isolated ventricular myocytes (Figure 1A through 1C). By contrast, no $I_{Ca,L}$ were recorded in adult ventricular myocytes from wild-type littermate (WT) hearts (Figure 1A). To determine the role played by T-type Ca\textsuperscript{2+} channels in the development of malignant arrhythmias and sudden death and to assess the potential therapeutic effect of T-type Ca\textsuperscript{2+} channel blockade in dnNRSF-Tg mice, we administered subpressor doses of efonidipine, a dual T- and L-type dihydropyridine Ca\textsuperscript{2+} channel blocker, or nitrendipine, a more L-type-selective dihydropyridine Ca\textsuperscript{2+} channel blocker, to dnNRSF-Tg mice for 7 weeks, beginning when they were 8 weeks of age. Initially, we confirmed that efonidipine significantly blocked $I_{Ca,T}$ in ventricular myocytes from dnNRSF-Tg mice (Figure 1D and 1E). Consistent with previous reports, efonidipine also blocked $I_{Ca,L}$ in those cells (Figure 1D and 1E). As shown in Figure 2A, efonidipine dramatically improved the survival rate among dnNRSF-Tg mice compared with mice treated with nitrendipine or control vehicle. We found that heart weight–to–body weight ratios and lung weight–to–body weight ratios did not differ among the control, efonidipine, and nitrendipine groups (Figure 2B and 2C). In addition, echocardiographic, hemodynamic, and histological analyses showed no significant differences among these 3 groups (Figure 2D, 2E, and 2F; Table). Consistent with these findings, there also was no significant difference in the expression of 2 cardiac stress marker genes, BNP and SERCA2, among the 3 groups (Figure 2G and 2H). Both efonidipine and nitrendipine modestly reduced the increase in CACNA1H expression seen in dnNRSF-Tg hearts (Figure 2I), and the expression of CACNA1G did not significantly differ among WT and dnNRSF-Tg mice (Figure 2J). These data suggest that efonidipine directly suppresses sudden death in dnNRSF-Tg mice without significantly affecting cardiac structure or function.

**Efonidipine Reduces Arrhythmogenicity in dnNRSF-Tg Mice**

We next used a telemetric monitoring system to examine the effects of each drug on ECG parameters in dnNRSF-Tg mice. We found that only efonidipine significantly suppressed the
number of premature ventricular contractions in dnNRSF-Tg hearts (Figure 3A). More importantly, it dramatically reduced the number of episodes of ventricular tachycardia (VT; Figure 3B).

We next assessed the effect of each drug on arrhythmogenicity in dnNRSF-Tg mice by performing an in vivo intracardiac electrophysiological analysis.14,25 We found that control dnNRSF-Tg mice were highly susceptible to induction of VT, as reported previously14 (Figure 3C and 3D), and that nitrendipine did not reduce that susceptibility (Figure 3D). By contrast, efonidipine significantly reduced the frequency of induced VT (Figure 3C and 3D). To confirm that inhibition of T-type Ca\(^{2+}\) currents is responsible for the suppression of arrhythmogenicity in dnNRSF-Tg hearts, we next treated dnNRSF-Tg mice with another dual T- and L-type Ca\(^{2+}\) channel blocker, mibefradil.28 After 1 week of treatment with mibefradil or efonidipine, dnNRSF-Tg mice showed significantly reduced susceptibility to induced VT (Figure 3E).

We next examined the effects of long-term drug treatment on the electrophysiological properties of myocytes isolated from dnNRSF-Tg hearts. When we measured action potentials elicited in isolated ventricular myocytes from WT and dnNRSF-Tg hearts, we found that in the latter, the membrane potential was somewhat depolarized, and the action potential duration was increased (Figure 3F and 3G). Efonidipine, but not nitrendipine, significantly restored the resting membrane potential in dnNRSF-Tg myocytes (Figure 3F and 3G).

### Table. Body Weight, Blood Pressure, and Hemodynamic Parameters in 12-Week-Old WT and dnNRSF-Tg Mice Treated With or Without Efonidipine or Nitrendipine

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<tr>
<th></th>
<th>WT</th>
<th>Untreated</th>
<th>Treated With Efonidipine</th>
<th>Treated With Nitrendipine</th>
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<td>Body weight, g</td>
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<td>LVDs, mm</td>
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<td>4.1±0.2*</td>
<td>3.8±0.1*</td>
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<td>FS, %</td>
<td>23.5±0.3</td>
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<tr>
<td>LVSP, mm Hg</td>
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<td>107±3*</td>
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<td>103±3*</td>
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<td>LVEDP, mm Hg</td>
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<td>dP/dt, mm Hg/s</td>
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<td>4683±192*</td>
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<td>−dP/dt, mm Hg/s</td>
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<td>HR, bpm</td>
<td>452±39</td>
<td>415±20</td>
<td>419±36</td>
<td>428±26</td>
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LVDd indicates left ventricular diastolic dimension; LVDs, left ventricular systolic dimension; FS, fractional shortening; IVS, intraventricular septum wall thickness; PW, posterior wall thickness; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; and HR, heart rate.

Values are mean±SEM. Numbers of mice tested were as follows: Body weight, n=10 for each group; blood pressure, n=6 for WT mice, n=14 for dnNRSF-Tg mice without drugs, n=5 for dnNRSF-Tg mice treated with efonidipine, n=7 for dnNRSF-Tg mice treated with nitrendipine; echocardiographic data, n=4 for WT mice, n=5 for dnNRSF-Tg mice without drugs, n=6 for dnNRSF-Tg mice treated with efonidipine, n=5 for dnNRSF-Tg mice treated with nitrendipine; hemodynamic data, n=5 for WT mice, n=4 for dnNRSF-Tg mice without drugs, n=6 for dnNRSF-Tg mice treated with efonidipine, n=5 for dnNRSF-Tg mice treated with nitrendipine.

*P<0.05 vs WT mice.

### Efonidipine Improves Cardiac Autonomic Nervous System Function in dnNRSF-Tg Mice

A disturbance of autonomic nerve activity that leads to increased sympathetic nerve activity and reduced parasympathetic nerve activity is involved in the increased arrhythmogenicity seen in patients with chronic heart failure. Heart rate variability (HRV) is a widely accepted index of cardiac autonomic nervous system activity.29 A previous frequency-domain analysis of HRV revealed that patients with severe heart failure show a progressive reduction in power in both the low-frequency and high-frequency ranges.29,30 Moreover, the reduction in low-frequency power is a significant predictor of sudden cardiac death in patients with heart failure.31,32

T-type Ca\(^{2+}\) channels are normally expressed in neuronal and endocrine tissues, where they play an important role in mediating neurotransmitter release and in the secretion of various neurohormonal factors, including catecholamines.33 Indeed, T-type Ca\(^{2+}\) channel blockade reportedly modulates autonomic activity.34,35 With that in mind, we hypothesized that in addition to its direct effects on cardiac electrophysiological properties, T-type Ca\(^{2+}\) blockade reduces arrhythmogenicity by modulating autonomic nerve function. To test that idea, we used HRV as an index with which to evaluate cardiac autonomic function in WT and dnNRSF-Tg mice.29 In mice, HRV predominantly correlates with parasympathetic activity.36 Mice are nocturnal, so that for any given “day,” the power in both the low- and high-frequency ranges was lower during the dark (night) phase, when the mice were more
active, than during the light (day) phase (online-only Data Supplement Figure IA and IB). In dnNRSF-Tg mice, the incidences of both premature ventricular contractions and VTs were much greater during the dark phase, which suggests the involvement of autonomic nerve activity in the generation of arrhythmias in these mice (online-only Data Supplement Figure IC and ID). In addition, the averages of both the low- and high-frequency powers over 24 hours in dnNRSF-Tg mice were markedly lower than in WT mice, which indicates a general reduction in parasympathetic activity in dnNRSF-Tg mice (Figure 4A, 4B, and 4C). Efonidipine dramatically increased the power in both the low- and high-frequency ranges of HRV in dnNRSF-Tg mice, whereas nitrendipine had little effect on HRV (Figure 4A, 4B, and 4C). We also found that urinary excretion of norepinephrine, which is indicative of the level of sympathetic nerve activity, was significantly higher in dnNRSF-Tg than in WT mice (Figure 5A). Moreover, the increased excretion of norepinephrine seen in dnNRSF-Tg mice was attenuated significantly only by efonidipine (Figure 5A).

We next evaluated the response of dnNRSF-Tg myocytes to catecholaminergic stimulation. We found that in the presence of isoproterenol 3 μmol/L, isolated ventricular myocytes from dnNRSF-Tg hearts showed early afterdepolarizations and spontaneous action potentials, whereas myocytes from WT hearts did not (Figure 5B, through 5D). In addition, systemic administration of isoproterenol induced VT more frequently in dnNRSF-Tg mice than in WT mice (Figure 5E). These data support our idea that abnormal autonomic nervous system balance, with decreased parasympathetic activity and increased sympathetic activity, facilitates arrhythmogenesis in dnNRSF-Tg mice. The increase in the frequency of isoproterenol-induced VT seen in dnNRSF-Tg mice was attenuated significantly by efonidipine but not by nitrendipine (Figure 5E). Thus, along with its direct effect, which reduces the vulnerability of the heart to arrhythmogenic stress (Figures 3D, 3E, and 5E), efonidipine also improves the cardiac autonomic nervous system balance, which further contributes to the suppression of lethal arrhythmias in dnNRSF-Tg mice.

**R(−)-Efonidipine, a Highly Selective T-Type Ca^{2+} Channel Blocker, Dramatically Improves Survival Among dnNRSF-Tg Mice**

Recently, R(−)-efonidipine was shown to be a specific blocker of T-type Ca^{2+} channels.17,18 To further confirm the
beneficial effects of T-type Ca\textsuperscript{2+} channel blockade in the prevention of sudden death in dnNRSF-Tg mice, we administered R-efonidipine (200 mg · kg\textsuperscript{-1} · d\textsuperscript{-1} PO) to dnNRSF-Tg mice for 20 weeks. We found that R-efonidipine did not significantly affect blood pressure, heart rate, cardiac structure, or systolic function in either WT or dnNRSF-Tg mice compared with vehicle (Figure 6A through 6E). By contrast, R(-)-efonidipine dramatically improved the survival rate among dnNRSF-Tg mice, which clearly suggests that T-type Ca\textsuperscript{2+} channel blockade prevents sudden death in dnNRSF-Tg mice (Figure 6F).

Efonidipine Reduces Sudden Death and Arrhythmogenicity in Mice With Acute Myocardial Infarction

Disturbance of cardiac autonomic nervous activity contributes to the incidence of arrhythmogenicity and mortality among patients with chronic heart failure due to nonischemic or ischemic cardiomyopathy, as well as among patients with acute myocardial infarction.\textsuperscript{29} To test whether efonidipine can improve the survival rate among mice with a cardiomyopathy other than the nonischemic cardiomyopathy seen in dnNRSF-Tg mice, we administered efonidipine or nitrendipine to WT mice previously subjected to acute myocardial infarction. We found that efonidipine significantly reduced the incidence of sudden death during the subacute phase of myocardial infarction (Figure 7A), although blood pressure, cardiac systolic function, and cardiac structure were all similar in the control, efonidipine, and nitrendipine groups (Figure 7B through 7F). When we further assessed arrhythmogenicity among these mice, we found that control mice with myocardial infarction were highly susceptible to induction of VT and that efonidipine, but not nitrendipine, significantly reduced the frequency of induced VT among mice with myocardial infarction (Figure 7G; online-only Data Supplement Figure II).

Discussion

Ca\textsuperscript{2+} influx is involved in multiple cellular processes, including cell growth, differentiation, and death. In cardiac myocytes, Ca\textsuperscript{2+} influx plays important roles under both normal physiological and pathophysiological conditions. One of the major sources of Ca\textsuperscript{2+} influx in excitable cells is voltage-gated Ca\textsuperscript{2+} channels, which have been classified into several

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Figure 4. Efonidipine restores cardiac autonomic nervous system function in dnNRSF-Tg mice. A, Representative HRV data from 12-week-old WT and dnNRSF-Tg (Tg) mice treated with or without efonidipine (Efo). Low-frequency (LF) and high-frequency (HF) ranges are shown. B and C, Average power of the LF (B) and HF (C) components of HRV recorded over a 24-hour period in WT and Tg mice treated with or without Efo or nitrendipine (Nit). \textit{P}<0.05 (n=5 for WT, n=4 for Tg without drugs, n=4 for Tg with Efo, n=4 for Tg with Nit).

Figure 5. Catecholamine-induced ventricular arrhythmias in dnNRSF-Tg hearts. A, Urinary norepinephrine (noradrenalin) levels in WT and dnNRSF-Tg (Tg) mice treated with or without efonidipine (Efo) or nitrendipine (Nit). \textit{P}<0.05 vs WT, \textit{P}<0.05 vs Tg treated with Efo (n=4 for WT, n=6 for Tg without drugs, n=4 for Tg with Efo, n=4 for Tg with Nit). B and C, Representative tracings of action potentials recorded in the presence (red lines) or absence (black lines) of isoproterenol 3 μmol/L from ventricular myocytes isolated from WT (B) and Tg (C) hearts. D, Representative trace showing normal and induced spontaneous action potentials recorded in the presence of isoproterenol 3 μmol/L from ventricular myocytes isolated from Tg hearts. Arrows indicate induced spontaneous action potentials. E, Number of episodes of VT induced in 15 minutes after intraperitoneal administration of isoproterenol (20 μg) to WT or Tg mice treated with or without Efo or Nit. \textit{P}<0.05 vs WT, \textit{P}<0.05 vs Tg with Efo. Numbers of mice tested were as follows: WT=5, Tg without drugs=5, Tg with Efo=5, Tg with Nit=4.
T-type Ca\textsuperscript{2+} channels are crucially involved in excitation-contraction coupling.\textsuperscript{37} Channels predominate in mature cardiac myocytes and are expressed abundantly in embryonic ventricular myocytes.\textsuperscript{4,5} After birth, however, expression of T-type Ca\textsuperscript{2+} channels is downregulated in ventricular myocytes,\textsuperscript{4,5} so that they are restricted to the conduction system,\textsuperscript{3,5} where they modulate pacemaking activities.\textsuperscript{3,38} But under conditions of cardiac hypertrophy and heart failure, T-type Ca\textsuperscript{2+} channels are reexpressed in ventricular myocytes.\textsuperscript{4,6–8}

Figure 6. \textit{R}(-)-efonidipine prolongs survival among dnNRSF-Tg mice. A and B, Blood pressures (A) and heart rates (B) in 12-week-old WT and dnNRSF-Tg (Tg) mice treated for 4 weeks with or without \textit{R}(-)-efonidipine [\textit{R}(-)]; \textit{n}=2 for WT, \textit{n}=3 for WT with \textit{R}(-), \textit{n}=5 for Tg, \textit{n}=4 for Tg with \textit{R}(-). C, D, and E, Left ventricular diastolic dimension (LVDd; C), \% fractional shortening (FS; E), and posterior wall thickness (PWT; F) assessed echocardiographically in sham and MI mice treated for 4 weeks with or without \textit{R}(-) [\textit{n}=2 for WT, \textit{n}=3 for WT with \textit{R}(-), \textit{n}=4 for Tg and Tg with \textit{R}(-)]. Two-way ANOVA revealed that Tg mice showed decreased blood pressure and enlarged LVDd compared with WT, and \textit{R}(-) had no effect on blood pressure, heart rate, or echocardiographic data. No mice status/medication status interaction was observed [in A, \textit{P}<0.001 between WT and Tg, \textit{P}=0.569 between without and with \textit{R}(-), interaction \textit{P}=0.267; in B, \textit{P}<0.725 between WT and Tg, \textit{P}=0.216 between without and with \textit{R}(-), interaction \textit{P}=0.179; in C, \textit{P}<0.001 between WT and Tg, \textit{P}=0.710 between without and with \textit{R}(-), interaction \textit{P}=0.131; in D, \textit{P}<0.001 between WT and Tg, \textit{P}=0.919 between without and with \textit{R}(-), interaction \textit{P}=0.457; in E, \textit{P}=0.304 between WT and Tg, \textit{P}=0.664 between without and with \textit{R}(-), interaction \textit{P}=0.950]. F, Kaplan-Meier survival curves for WT and Tg mice with or without \textit{R}(-) during a 20-week drug administration period beginning at 8 weeks of age. *\textit{P}<0.05 [\textit{n}=6 for WT, \textit{n}=3 for WT with \textit{R}(-), \textit{n}=13 for Tg, \textit{n}=9 for Tg with \textit{R}(-)].

Figure 7. Efonidipine prevented sudden death among mice with acute myocardial infarction. A, Kaplan-Meier survival curves for sham-operated mice (sham) and mice with myocardial infarction (MI) treated for 30 days with or without efonidipine (Efo) or nitrendipine (Nit) beginning at 8 to 12 weeks of age. *\textit{P}<0.05 (\textit{n}=12 for sham, \textit{n}=39 for MI, \textit{n}=29 for MI with Efo, and \textit{n}=29 for MI with Nit). B and C, Blood pressures (B) and heart rates (C) in sham and MI mice treated for 4 weeks with or without Efo or Nit. *\textit{P}<0.05 vs sham (\textit{n}=7 in each group). D, E, and F, Left ventricular diastolic dimension (LVDd; D), \% fractional shortening (FS; E), and posterior wall thickness (PWT; F) assessed echocardiographically in sham and MI mice treated for 4 weeks with or without Efo or Nit. *\textit{P}<0.05 vs sham (\textit{n}=10 for sham, \textit{n}=6 for MI, \textit{n}=10 for MI with Efo, and \textit{n}=8 for MI with Nit). G, Frequency of mice with inducible VT in sham and MI mice treated for 4 weeks with or without Efo or Nit are shown. VT indicates number of mice with inducible VT; total, total number of mice tested. *\textit{P}<0.05 vs WT, †\textit{P}<0.05 vs MI with Efo.
and they are thought to be involved in the altered cardiac function and arrhythmogenicity seen in the diseased myocardium. Consistent with that idea, the dual T- and L-type Ca\textsuperscript{2+} channel blocker mibebradil attenuates the pathological processes seen in some animal models of cardiac disease. Moreover, it was recently reported that genetic deletion of CACNA1H results in resistance to pathological cardiac hypertrophy. Nonetheless, the effect of T-type Ca\textsuperscript{2+} channel blockade on the incidence of malignant arrhythmias and sudden death remains unknown.

In the present study, we demonstrated that the dual T- and L-type Ca\textsuperscript{2+} channel blocker efonidipine prevents the sudden death and arrhythmogenicity otherwise seen in dnNRSF-Tg mice, whereas nitrrendipine, a selective L-type Ca\textsuperscript{2+} channel blocker, does not. Suppression of arrhythmogenicity also was observed when dnNRSF-Tg mice were treated with mibebradil. Although the doses of nitrrendipine used in the present studies (20 and 60 mg/kg) did not significantly affect blood pressure in dnNRSF-Tg mice, those doses previously were shown to effectively block L-type Ca\textsuperscript{2+} channels in mice. We also observed that treatment of db/db mice, a mouse model of type 2 diabetes–associated hypertension, with nitrrendipine 10 mg · kg\textsuperscript{-1} · d\textsuperscript{-1} for 8 days significantly reduced their elevated blood pressure (unpublished observation). These results clearly demonstrate that blockade of T-type Ca\textsuperscript{2+} channels, not L-type Ca\textsuperscript{2+} channels, mediates the effects of efonidipine and mibebradil on dnNRSF-Tg mice, although there is still the possibility that unknown effects of efonidipine and mibebradil on other currents are responsible. Furthermore, R(−)-efonidipine, a recently identified specific blocker of T-type Ca\textsuperscript{2+} channels, dramatically improved the survival rate among dnNRSF-Tg mice, which strongly supports the notion that T-type Ca\textsuperscript{2+} channels play a key role in mediating the lethal arrhythmias seen in this animal model. In addition, efonidipine, but not nitrrendipine, also reduced the incidence of sudden death and arrhythmias among mice with acute myocardial infarction. Collectively, these results clearly demonstrate that blockade of T-type Ca\textsuperscript{2+} channel blockers may correct abnormalities in ventricular electrophysiology in part by inhibiting these pathological signaling pathways.

Disturbance of cardiac autonomic nervous activity that leads to increased sympathetic nerve activity and decreased parasympathetic nerve activity contributes to the increased arrhythmogenicity seen in patients with chronic heart failure. HRV analysis is widely used to assess autonomic nerve function in the heart and has been shown to correlate with the prognosis of patients with heart failure. Consistent with that idea, we observed in the present study that efonidipine significantly reduces the incidence of sudden death among mice with acute myocardial infarction further support the idea that correcting the balance between sympathetic and parasympathetic nerve activity affects the electrophysiological properties of ventricular myocytes. The present results showing that efonidipine reduces the incidence of sudden death among mice with acute myocardial infarction further support the idea that correcting the balance in cardiac autonomic nerve activity through blockade of T-type Ca\textsuperscript{2+} channels contributes to the prevention of malignant arrhythmias and sudden death in dnNRSF-Tg mice. There is also the possibility that correcting the balance between sympathetic and parasympathetic nerve activity affects the electrophysiological properties of ventricular myocytes. The present results showing that efonidipine reduces the incidence of sudden death among mice with acute myocardial infarction further support the idea that correcting the balance in cardiac autonomic nerve activity through blockade of T-type Ca\textsuperscript{2+} channels contributes to the prevention of malignant arrhythmias and sudden death in dnNRSF-Tg mice. However, disturbances in cardiac autonomic nervous activity are associated with sudden death in patients with acute myocardial infarction.

Mibebradil was approved for use in the treatment of hypertension, angina pectoris, and congestive heart failure in 1997 but was withdrawn from the market because of an unexpected side effect unrelated to the T-type Ca\textsuperscript{2+} channel blockade: It inhibited cytochrome p450s, thereby causing negative drug-drug interactions.
T-type Ca\(^{2+}\) Channels and Arrhythmia

Kinoshi et al

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**CLINICAL PERSPECTIVE**

Despite recent progress in nonpharmacological therapy, pharmacological interventions for the treatment and prevention of lethal arrhythmias associated with heart failure remain limited. In this study, we used mouse models of ischemic and nonischemic cardiomyopathy to show that T-type Ca\(^{2+}\) channel blockade diminishes arrhythmogenicity and prevents sudden death in heart failure. The dual T- and L-type Ca\(^{2+}\) channel blocker mibefradil was withdrawn from the market because of an unexpected side effect unrelated to the T-type Ca\(^{2+}\) channel blockade: It inhibited cytochrome p450s, thereby causing negative drug-drug interactions. Another dual T- and L-type Ca\(^{2+}\) channel blocker, efonidipine, which we used in this study, has been used in Japan for several years to treat hypertension, and no severe side effects have yet been identified. Although further investigation is necessary, our results suggest that efonidipine and perhaps other T-type Ca\(^{2+}\) channel blockers, especially selective T-type Ca\(^{2+}\) channel blockers such as the R(−)-isomer efonidipine, may be clinically useful for the prevention of lethal arrhythmias and sudden death in patients with heart failure.
SUPPLEMENTAL MATERIAL

Supplemental Methods

Animal experiments

Animal care and all experimental protocols were conducted in accordance with the institutional guidelines of Kyoto University Graduate School of Medicine. Beginning at 8 weeks of age, dnNRSF-Tg mice were left untreated (control) or were treated for 7 weeks with efonidipine (40 mg/kg/day P.O.) or nitrendipine (20 mg/kg/day P.O.). In another experiment, 10- or 11-week-old dnNRSF-Tg mice were left untreated (control) or treated for 7 days with mibefradil (15 mg/kg/day), efonidipine hydrochloride (200 mg/kg/day) or nitrendipine (60 mg/kg/day). Efondipine or nitrendipine was given as food admix in normal mice chow. Mibefradil was dissolved in water to a concentration of 2 mg/ml, and was given daily via gastric gavage adjusted to the individual body weight of each mouse. The same amount of water was given to the other treatment groups in the same manner. The doses of mibefradil, efondipine and nitrendipine were chosen based to earlier reports and our preliminary studies.

In the experiment using $R(\cdot)$-efondipine, beginning at 8 weeks of age, dnNRSF-Tg mice were left untreated (control) or were treated for 20 weeks with $R(\cdot)$-isomer efondipine (200 mg/kg/day P.O.). $R(\cdot)$-efondipine was given as a food admix in normal mice chow.
At 12 weeks of age, we analyzed blood pressures, heart rates, and cardiac function.

Acute myocardial infarction was induced in female C57BL/6 mice (age, 8 to 12 weeks; weight, 19 to 24 g) by ligation of the left coronary artery as previously described\(^5\). The same procedure without coronary artery ligation served as the sham operation. Beginning 1 day after the operation, mice were left untreated (control) or were treated for 30 days with efonidipine hydrochloride (200 mg/kg/day) or nitrendipine (60 mg/kg/day), which were given as a food admix in normal mouse chow.

Efonidipine and \(R\)(-)-isomer efonidipine were supplied by Nissan Chemical Industries, Ltd. (Tokyo, Japan). Nitrendipine was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

**Patch clamp studies**

Myocytes were dispersed using an improved method described previously\(^6\). To record T- and L-type Ca\(^{2+}\) currents, electrodes were filled with Cs\(^+\)-rich solution containing (in mM): 100 CsCl, 50 NMDG, 10 TEA, 5 MgATP, 5 HEPES and 10 EGTA (pH=7.2 with CsOH). After establishment of the ruptured whole cell patch configuration in normal Tyrode solution, the bathing solution was switched to Na\(^+\)-free solution containing (in mM): 140 NMDG, 5 CsCl, 5 4-AP, 1.8 CaCl\(_2\), 0.5 MgCl\(_2\), HEPES 5 (pH=7.4 with CsOH). A stock solution of 10 mM efonidipine in DMSO was diluted to the desired concentration using Na\(^+\)-free bathing
solution (final concentration, 10 μM).

**Intracardiac electrophysiology**

Mice were intubated and anesthetized with 0.5-1.5% isoflurane, after which surface electrocardiography leads (limb leads) were placed. Then using a 1.7 French octapolar catheter (CIBer mouse EP, NuMe, Hopkinton, New York, USA) inserted via the jugular vein, a standard electrophysiological study protocol was performed as described previously\(^7\). Rapid ventricular pacing using the extrastimulation (S\(_1\)S\(_2\)) technique was carried out with 1-2 extra stimuli to determine the ventricular refractory period and to attempt induction of ventricular arrhythmias. The stimulation was administered at twice the ventricular diastolic capture threshold.

**Noninvasive Blood Pressure Measurements**

Systolic Blood Pressure (SBP) were measured in conscious mice using the tail-cuff method (Softron Ck Ltd) as described previously\(^7\).

**Echocardiographic and hemodynamic analysis**

Echocardiography was performed using an echocardiography system (Toshiba power vision 8000) equipped with a 12-MHz imaging transducer as described previously\(^7\).
**Histological Examination**

Hearts were fixed in 10% formalin and prepared for histological analysis as described previously.  

**Quantitative RT-PCR analysis**

Using 50 ng of total RNA prepared from ventricles, levels of mouse *BNP, NRSF, SERCA2, CACNA1H, CACNA1G* and *GAPDH* mRNA were determined by quantitative real-time PCR according to the manufacture’s protocol (Applied Biosystems, Zaventam, Belgium), as previously described. The sequences of the forward and reverse primers and the probes with fluorescent dye (FAM) and quencher (TAMRA) were also described previously.  

**Systemic administration of isoproterenol**

Mice were intubated and anesthetized with 0.5-1.5% isoflurane, after which surface electrocardiography leads (limb leads) were placed, and 20 μg of isoproterenol were injected intraperitoneally. The number of episodes of ventricular tachycardia (VT) that occurred during the 15 min period immediately after injection was then calculated.
Measurement of urinary noradrenalin excretion

For analysis of urinary noradrenalin excretion, 12-week-old wild type (WT) and dnNRSF-Tg mice from each treatment group were placed in metabolic cages and given free access to food and water. Urinary samples were then collected from 10 o’clock at night to 9 o’clock in the morning. Urinary noradrenalin excretion was assessed using reverse phase high-performance liquid chromatography.

Analysis of electrocardiographs by telemetry

To monitor ambulatory electrocardiographs, radio frequency transmitters (TA 10ETA-F20; Data Science, St Paul, Minnesota, USA) were implanted as previously described.

Heart Rate Variability

Fifteen-minute periods of electrocardiographic data with little noise and few ectopic beats were collected from day 3 to day 5 after implantation of radio frequency transmitters and then analyzed for heart rate variability (HRV). Because of the circadian rhythm of HRV, four data sets obtained during periods extending from 7-12, 13-18, 19-24 and 1-6 o’clock were averaged for each mouse. Spectral analysis of the heart rate recordings using a fast Fourier transform (FFT) algorithm on sequences of 1024 points was carried out using HEM 3.4 software (Notocord Systems). Cut-off frequencies for power in the low-frequency (LF: 0.15
to 1.5 Hz) and high-frequency (HF: 1.5 to 5.0 Hz) ranges were based on previous experiments with mice\textsuperscript{10}. After FFT analysis, the data that contained ectopic beats or arrhythmic events were deleted manually. In mice, HRV predominantly correlates with parasympathetic activity\textsuperscript{10}. Consistent with that earlier finding, we observed that a muscarinic receptor blocker (atropine) but not a β-adrenergic receptor blocker (propranolol) reduces HRV in both the LF and HF ranges in WT and dnNRSF-Tg mice (unpublished data).
## Supplemental Tables

### Supplemental Table 1. Parameters in WT and dnNRSF-Tg mice treated with or without efonidipine or nitrendipine, shown in Figure 2-5

<table>
<thead>
<tr>
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<th>WT</th>
<th>dnNRSF-Tg</th>
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<tr>
<td>Fig.2B HW/BW (mg/g)</td>
<td>5.0±0.1</td>
<td>6.1±0.2*</td>
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<tr>
<td>Fig.2C LW/BW (mg/g)</td>
<td>6.1±0.2</td>
<td>6.4±0.3</td>
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### Echographic data

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<td></td>
<td>Fig.2D LVDD ; 8W (mm)</td>
<td>3.9±0.1</td>
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<td>Fig.2D LVDD ; 10W (mm)</td>
<td>4.1±0.2</td>
<td>4.5±0.1</td>
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<td>Fig.2D LVDD ; 12W (mm)</td>
<td>4.1±0.0</td>
<td>4.8±0.2</td>
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<td>Fig.2E FS ; 8W (%)</td>
<td>29.3±2.3</td>
<td>24.4±1.4</td>
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<td>Fig.2E FS ; 10W (%)</td>
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<td>Fig.2E FS ; 12W (%)</td>
<td>23.5±0.3</td>
<td>14.0±1.6</td>
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### RT-PCR

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<tr>
<td></td>
<td>Fig.2G BNP</td>
<td>1.00±0.13</td>
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<td>Fig.2H SERCA2</td>
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<td>Fig.2I CACNA1H</td>
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<td>Fig.2J CACNA1G</td>
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### ECG monitoring

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<tr>
<td></td>
<td>Fig.3A PVC (/h)</td>
<td>0</td>
<td>861.3±134.5‡</td>
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<td>Fig.3B VT (/h)</td>
<td>0</td>
<td>22.7±2.9‡</td>
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<td>Fig.3G Resting membrane potential</td>
<td>-82.72±0.84</td>
<td>-75.88±0.71*</td>
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### Heart rate variability

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<td></td>
<td>Fig.4F LF power (ms)</td>
<td>5.69±0.80</td>
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<td>Fig.4G HF power (ms)</td>
<td>1.04±0.34</td>
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<td>Fig.5A Urinary Noradrenalin</td>
<td>3.0±1.6</td>
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<td>Fig.5E Isoproterenol-induced VT</td>
<td>0.8±0.8</td>
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Values are means ± SEM; *p<0.05 vs. WT, †p<0.05 vs. Tg, ‡p<0.05 vs. Tg+Efo. HW/BW, Heart weight-to-body weight ratios; LW/BW, lung weight-to-body weight ratios; LVDD, left ventricular diastolic dimension; FS, fractional shortening; PVC, premature ventricular contraction; VT, ventricular tachyarrhythmia; LF, Low frequency; HF, high frequency.
Supplemental Table 2. Parameters in WT and dnNRSF-Tg mice treated with or without \(R(-)-\)efonidipine, shown in Figure 6

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<td>- R(-)</td>
<td>- R(-)</td>
<td>- R(-)</td>
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<tr>
<td>Blood pressure (mmHg)</td>
<td>113.0±1.0</td>
<td>112.0±1.5</td>
<td>101.0±1.1</td>
<td>104.0±2.0</td>
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<td>Heart rate (/min)</td>
<td>608.0±72.0</td>
<td>516.0±32.3</td>
<td>566.6±54.8</td>
<td>519.3±28.9</td>
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<td>LVDd (mm)</td>
<td>3.5±0.2</td>
<td>3.7±0.1</td>
<td>4.3±0.1</td>
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<td>FS (%)</td>
<td>40.0±1.0</td>
<td>38.7±0.3</td>
<td>26.5±2.6</td>
<td>28.3±1.4</td>
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<td>PWTh (mm)</td>
<td>0.75±0.05</td>
<td>0.73±0.04</td>
<td>0.71±0.02</td>
<td>0.70±0.02</td>
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Values are means ± SEM. LVDd, left ventricular diastolic dimension; FS, fractional shortening; PWTh, posterior wall thickness.
Supplemental Table 3. Parameters in mice with acute myocardial infarction treated with or without efonidipine or nitrendipine, shown in Figure 7.

<table>
<thead>
<tr>
<th></th>
<th>sham</th>
<th>MI</th>
<th>+ Efo</th>
<th>+ Nit</th>
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<tr>
<td><strong>Fig.7B</strong> Blood Pressure (mmHg)</td>
<td>114.4±1.8</td>
<td>104.4±2.1*</td>
<td>106.1±2.8*</td>
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<td><strong>Fig.7C</strong> Heart Rate (/min)</td>
<td>561.0±23.6</td>
<td>552.7±19.1</td>
<td>544.3±22.6</td>
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<td><strong>Fig.7D</strong> LVDd (mm)</td>
<td>3.5±0.0</td>
<td>4.9±0.4*</td>
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<td><strong>Fig.7E</strong> FS (%)</td>
<td>40.6±1.4</td>
<td>17.4±1.1*</td>
<td>15.6±1.0*</td>
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<td><strong>Fig.7F</strong> PWTh (mm)</td>
<td>0.80±0.02</td>
<td>0.70±0.06</td>
<td>0.80±0.04</td>
<td>0.90±0.05</td>
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Values are means ± SEM; *p<0.05 vs. sham. LVDd, left ventricular diastolic dimension; FS, fractional shortening; PWTh, posterior wall thickness.
Suppl. Fig2

sham
SECG
RV-IECG
RA-IECG

VT (+)

MI
SECG
RV-IECG
RA-IECG

VT (+)

MI+Efo
SECG
RV-IECG
RA-IECG

VT (-)

MI+Nit
SECG
RV-IECG
RA-IECG

VT (+)
Supplemental Figure legends

**Figure 1.** A and B, The power of the low frequency (LF) (A) and high frequency (HF) (B) components of HRV in WT mice over the course of a 24-h period (n=6). C and D, The numbers of PVC (C) and VT (D) in dnNRSF-Tg mice during a 24 h period (n=6). (A-D) The white bars show the light phase, the black bars show the dark phase.

**Figure 2.** Efonidipine reduces arrhythmogenicity in infarcted hearts. A, Representative ECG traces from an *in vivo* electrophysiological study carried out to evaluate the inducibility of VT in infarcted hearts from mice treated with or without efonidipine (Efo) or nitrendipine (Nit).
Supplemental References


