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Slow progressive conduction and contraction defects in loss of Nkx2–5 mice after cardiomyocyte terminal differentiation

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Abstract

Mutations in homeoprotein NKX2–5 are linked to human congenital heart disease resulting in various cardiac anomalies, as well as postnatal progressive conduction defects and occasional left ventricular dysfunction, yet the function of Nkx2–5 in the postnatal period is largely unexplored. In the heart, the majority of cardiomyocytes are believed to complete cell-cycle withdrawal shortly after birth, which is generally accompanied by re-organization of chromatin structure demonstrated in other tissues. We reasoned that effects of loss of Nkx2–5 in mice may be different after cell-cycle withdrawal compared to perinatal loss of Nkx2–5, which results in rapid conduction and contraction defects within 4 days after deletion of Nkx2–5 alleles (Circ Res. 2008;103:580).

In this study, floxed-Nkx2–5 alleles were deleted using tamoxifen-inducible Cre transgene (Cre-ER^{TM}) beginning at 2 weeks of age. Loss of Nkx2–5 beginning at 2 weeks of age resulted in conduction and contraction defects similar to perinatal loss of Nkx2–5, however with substantially slower disease progression demonstrated by 1° atrioventricular block at 6 weeks of age (4 weeks after tamoxifen injections), and heart enlargement after 12 weeks of age (10 weeks after tamoxifen injections). The phenotypes were accompanied by slower and smaller degree of reduction of several critical Nkx2–5 downstream targets that were observed in mice with perinatal loss of

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Nkx2–5. These results suggest that Nkx2–5 is necessary for proper conduction and contraction after 2 weeks of age, but with substantially distinct level of necessity at 2 weeks of age compared to the perinatal period.

Introduction

Nkx2–5 is a homeodomain-containing transcription factor, highly conserved among species and one of the earliest cardiogenic markers1,2 with expression continuing throughout adulthood.3–5 Targeted disruption of Nkx2–5 in mice causes embryonic lethality around embryonic (E) day 10.5, with retarded cardiac development.6,7 Mice with ventricular-restricted Nkx2–5 deletion from E 8.0–8.5 survive but demonstrate progressive and advanced conduction defects as well as left ventricular hypertrophy postnatally.8 Heterozygous mutations in human NKX2–5 cause various cardiac anomalies, and postnatal progressive conduction defects and occasional left ventricular dysfunction.9–12

Nkx2–5 function has predominantly been studied in mouse embryos or stem cells when cardiomyocytes are proliferating.13,14 To address Nkx2–5 function at distinct stages of development, we recently generated tamoxifen-inducible Nkx2–5 knockout mice. Using mice with perinatal loss of Nkx2–5, we have demonstrated that Nkx2–5 is critically important for cardiac conduction and contraction in the perinatal period as well. In these mice, atrio-ventricular (AV) block and heart enlargement appeared within 4 days after tamoxifen injection, leading to premature death due to cardiac dysfunction.15 Rapid disease progression was accompanied by the rapid decrease of several gene products important for both conduction and contraction, including cardiac Na^+ channel $Na_v1.5(\alpha)$, ryanodine receptor 2 and cardiac myosin light chain kinase (MLCK).15,16

Our ongoing hypothesis is that Nkx2–5 actively regulates a critical set of genes in postnatal cardiomyocytes to maintain proper cardiac function. Less well understood is the function of Nkx2–5 from the time between perinatal development to adulthood. Recent studies demonstrate the formation of a population of new myocytes in the adult heart;17 although perhaps the majority of cardiomyocytes may be considered to lose their ability for proliferation (G1 cell-cycle arrest) and DNA synthesis during the first week after birth, and transit into cells with highly specialized structure and function.18 Because G1 cell-cycle arrest is generally linked to morphological, metabolic and gene expression changes with re-organization of nuclear architecture and chromatin structure,19,20 cardiomyocytes at G1 cell-cycle arrest may share these properties. We reasoned this transition might change the cardiomyocyte responses to cardiac transcription factors including Nkx2–5.

In this study, we deleted the Nkx2–5 gene beginning at 2 weeks of age, which resulted in a slow progressive conduction defect and heart enlargement, accompanied by a slow downregulation of critical genes that were important determinants for the phenotypes observed in perinatal loss of Nkx2–5 mice.

Materials and Methods

Inducible Nkx2–5 knockout mice

Floxed-Nkx2–5 mice8 were bred with transgenic mice carrying the Cre-ER™ gene under CMV promoter.21 Details for mouse generation were described previously.15 To delete the floxed-Nkx2–5 gene, tamoxifen (1 mg/g body weight, ip) was injected into mice beginning at 2 weeks of age for 4 consecutive days. All animal care protocols fully conformed to the Association for the Assessment and Accreditation of Laboratory Animal Care, with approvals from the University of Florida Institutional Animal Care and Use Committee.

Telemetry ECG recordings and echocardiogram

Recording of telemetry ECG (Data Sciences International) was performed 3 days after implantation of wireless radiofrequency telemetry devices to avoid effects of anesthesia on ECG. Telemetry data were analyzed using PowerLab software (ADInstruments) as described previously.22 M-mode ultrasound imaging of the left ventricle of anesthetized mice (pentobarbital 60 mg/kg, ip) was obtained at the level of the papillary muscle from a parasternal window using an ultrasound biomicroscope with a single transducer with a frequency 40 MHz (VisualSonics, Toronto, Canada).

Northern blotting, immunostaining and histological analyses

Northern blot analyses were performed using the following probes: Nkx2–5 coding probe, PflMI-EcoRI fragment of mouse Nkx2–5 cDNA; atrial natriuretic factor (ANF) (330 bp PCR products for rat ANF, F, 5′-GGGGTAGGATTGACAGGATTGG-3′; R, 5′-CCGTGGTGCTGAAGTTTATTCG-3′); brain natriuretic peptide (BNP) (429 bp PCR products, F, 5′-TGGGGAGGCGAGACAAGGG -3′; R, 5′-TCTTCCTACAACAACTTCAGTGCG -3′); β-myosin heavy chain (MHC)(oligonucleotide probe, 5′-GCTTTATTCTGCTTCCACCTAAAGGGCTGTTGCAAAGGCTCCAGGTCTGAGGGCTTC-3′); GAPDH (450 bp PCR products, F, 5′-TTCATTGACCTCAACTACAT-3′; R, 5′-GTGGCAGTGATGGCATGGAC-3′).

Immunostaining was performed with the following primary antibodies: anti-Nkx2–5 pAb,5 and sarcomeric actinin (Sigma A7811). Fluorescent microscopic images were obtained using ZEISS Axiovert200M with or without Apotome. Histological analyses, including hematoxylin/eosin and Masson's trichrome staining, acetylcholine esterase staining in frozen tissue sections and whole mount acetylcholine esterase staining were performed as described previously.15 Digitalized images from AV node, LV free wall in the longitudinal section at the level of nuclei, and isolated ventricular myocytes were utilized for measurement using Image J software as described previously.23–25

Simultaneous recording of cardiac contraction and Ca^{2+} measurements

Rod-shaped cardiomyocytes with clear cross striations, staircase ends and surface membranes free from blebs isolated from approximately 24 weeks old flox/flox or flox/flox/Cre mice were studied for simultaneous measurements of cardiomyocyte cell shortening and intracellular free calcium concentration as described previously.16

Real-time RT-PCR

Real-time RT-PCR was performed using inventoried Taqman Gene Expression Assays (Applied Biosystems): cardiac MLCK, Mm00615292; Scn5a, Mm00451971; RyR2, Mm0046587; ANF, Mm01255748; BNP, Mm00435304; βMHC (MHC7), Mm00600555. Data were normalized to β-actin expression (No. 4352933E). Duplicate experiments were averaged.

Statistical analysis

Values among groups were compared using ANOVA and Fisher PLSD post-hoc test (StatView version 5.01). $P<0.05$ was considered significant.

Results

Slow progressive conduction defects and heart enlargement in Nkx2–5 knockout mice from 2 weeks of age

Recently, we generated a mouse model to delete Nkx2–5 gene by tamoxifen injection15 by mating mice homozygous for floxed-Nkx2–5 alleles8 and heterozygous for the Cre-ER™ transgene under the control of the CMV enhancer and the chicken β-globin promoter.21 Tamoxifen-injection within 24 hr before birth induced rapid progressive conduction and contraction defects within 4 days, which leads to premature death.15

In this study, tamoxifen was injected at 2 weeks of age for four consecutive days. Time course studies at multiple time points are shown in Figure 1A. Measurement of heart weight/tibial length and telemetry ECG recordings of Nkx2–5-knockout mice were performed at multiple time points from 3.5 to 24 weeks of age (1.5 and 22 weeks from the initiation of tamoxifen injections respectively). Deletion of the Nkx2–5 gene followed by reduction of Nkx2–5 mRNA (Figure 1B) and proteins (Figure 1C) was demonstrated 1 day after four consecutive tamoxifen-injections (approximately 2.5 weeks of age), as well as 3 days (approximately 3 weeks of age) and 6 days (approximately 3.5 weeks of age) after injections. Reduction of Nkx2–5 mRNA continued to 24 weeks of age by tamoxifen-injections at 2 weeks of age as demonstrated in Northern blotting (Figure 1D).

Telemetry ECG recordings of Nkx2–5-knockout mice and tamoxifen-injected control mice (flox/flox or flox/wild/Cre) were performed at multiple time points from 3.5 to 24 weeks of age (1.5 and 22 weeks after tamoxifen injections respectively) (Table 1). Nkx2–5 knockout mice first demonstrated PR prolongation at 9 weeks of age accompanied by intermittent 2° AV block. The PR interval was progressively prolonged, and wide QRS was observed at 24 weeks of age (Table 1). With the exception of one mouse at 24 weeks of age with intermittent 2° AV block, heterozygous Nkx2–5 knockout mice (flox/wild/Cre) did not demonstrate PR prolongation or wide QRS (Table 1). Examples of telemetry ECG recordings normal at 3.5 weeks of age and with demonstration of 2° AV block and wide QRS at 24 weeks of age in Nkx2–5 knockout hearts are demonstrated in Figure 2A.

Heart weight/tibial length, an indicator of heart enlargement, was slightly but significantly increased in mice with Nkx2–5 knockout beginning at 12 weeks of age compared to

tamoxifen-injected control mice (flox/flox or flox/wild/Cre) (Figure 2B). A reduction of cardiac contractile performance (% fraction shortening), and an increase of left ventricular systolic dimension (LVDs) were observed in Nkx2–5 knockout mice at 24 weeks of age using 40 MHz ultrasound imaging (Figures 2C, D).

Taken together, mice with loss of Nkx2–5 from 2 weeks of age demonstrate conduction and contraction defects. Although this phenotype is qualitatively similar to the phenotype observed with perinatal loss of Nkx2–5,15 disease progression and severity are markedly distinct despite using the genetically identical mouse model. For instance, when tamoxifen was administered at 2 weeks of age, no phenotype was apparent over a time-span of 1.5 weeks after tamoxifen injection (3.5 weeks of age), in contrast to perinatal tamoxifen administration.15 In addition, mice with loss of Nkx2–5 from 2 weeks of age survive over 1.5 years of age despite the presence of PR-prolongation, wide QRS and slight increase of heart weight/tibial length (data not shown), while mice with perinatal loss of Nkx2–5 die prematurely.15

Reduced expression of Nkx2–5 protein in cardiomyocytes accompanied by small AV node

Immunostaining confirmed decreased Nkx2–5 expression in Nkx2–5 knockout ventricles at 3.5 weeks of age (Figure 3A, left panels). Despite slight heart enlargement in Nkx2–5 knockout mice at 24 weeks of age, interstitial fibrosis was not apparent in ventricles (Figure 3A, right panels). In adjacent tissue sections, including the acetylcholine esterase-positive AV node, positive Nkx2–5 staining was demonstrated in the control heart, but not in the Nkx2–5 knockout heart (Figure 3B). Although formation of the AV node is completed before birth, whole mount acetylcholine esterase staining demonstrated that AV nodal surface area size was smaller at 24 weeks of age in hearts with loss of Nkx2–5 beginning at 2 weeks of age (Figures 3C, D). This observation is similar to previous studies using ventricular-specific deletion of Nkx2–5 from the embryonic stage in MLC2v-Cre mice and in perinatal Nkx2–5 knockout mice;8 however, AV nodal fibrosis was not apparent in mice with Nkx2–5 knockout from perinatal15 and 2 weeks of age (Figure 3E).

In addition, nuclei in the AV node appeared more condensed and nuclear number/mm^2 was significantly increased in AV node in Nkx2–5 knockout vs. control hearts at 24 weeks of age (Figure 3E, F). To examine whether this could be due to reduced cardiomyocyte cell size in the AV node of Nkx2–5 knockout mice, cell diameter was measured in the longitudinal section at the position of nuclei. AV nodal cell width in control flox/flox mice was increased from 3.5 to 24 weeks of age, but was not increased in flox/flox/Cre mice, leading to smaller AV nodal cell width in Nkx2–5 knockout vs. control mice at 24 weeks of age (Figure 3G, upper panels; 3H, left panel).

In contrast, cell width in non-AV nodal myocytes in left ventricle was increased between 3.5 to 24 weeks of age in both Nkx2–5 knockout and control mice, and was slightly, but not significantly wider in Nkx2–5 knockout at 24 weeks of age (Figure 3G, lower panels; 3H, right panel). This observation was further examined using isolated cardiomyocyte measurements that demonstrated a significant increase in mean surface area, long axis and short axis length in Nkx2–5 knockout myocytes (Figures 4A–C). Taken together, Nkx2–5

knockout hearts at 24 weeks of age demonstrated ventricular myocyte hypertrophy, and a reduction in cardiomyocyte cell width in the AV node.

Nkx2–5 knockout ventricular cardiomyocytes are morphologically and functionally compensated

At 24 weeks of age, cardiomyocytes isolated from mice with Nkx2–5 knockout from 2 weeks of age did not show significant differences in fractional shortening, +dL/dT (rate of contraction) and −dL/dT (rate of relaxation) compared to cells from control mice at Ca^{2+} superfusate concentration of 1.2 mM (Figure 4D). Notably, Ca^{2+} transient amplitude and systolic fluorescence ratio were significantly lower in Nkx2–5 knockout cardiomyocytes (Figure 4D), which is similar to perinatal Nkx2–5 knockout cardiomyocytes with reduction of ryanodine receptor 2.15 When Ca^{2+} superfusate concentration was increased from 1.2 to 2.5 mM, the increase in fractional shortening was blunted in Nkx2–5 knockout cardiomyocytes compared to control cardiomyocytes (Figure 4E). These results indicate that cardiomyocytes from Nkx2–5 knockout hearts were morphologically and functionally compensated despite reduced Ca^{2+}-handling, and a reduction in contraction appeared only under conditions of increased demand.

Slow reduction of Nkx2–5 downstream targets in hearts with loss of Nkx2–5 from 2 weeks of age

Expression of cardiac hypertrophic markers, atrial natriuretic factor (ANF) and brain natriuretic peptide (BNP), is regulated by Nkx2–5 demonstrated in embryos7 as well as at PD4 in perinatal loss of Nkx2–5 (Figure 5A). In hearts from mice with Nkx2–5 knockout from 2 weeks of age, expression of ANF and BNP was also downregulated early after tamoxifen injections at 3.5 weeks of age (1.5 weeks after tamoxifen injection) and further decreased at 6 weeks of age (4 weeks after tamoxifen injection) compared to control flox/flox hearts (Figure 5B).

In contrast, expression of another hypertrophy ic marker, β-myosin heavy chain (βMHC) was induced 4 days after tamoxifen injection in hearts with perinatal loss of Nkx2–5 (Figure 5A); while it was barely detected at 3.5 weeks of age in mice with Nkx2–5 knockout from 2 weeks of age, but was upregulated at 6 weeks of age (Figure 5B). Thus, delayed downregulation of ANF and BNP as well as delayed upregulation of βMHC in hearts from mice with Nkx2–5 knockout from 2 weeks of age contrasts with the rapid changes in expression within 4 days after tamoxifen injection in hearts with perinatal loss of Nkx2–5. At 24 weeks of age when marked cardiomyocyte hypertrophy was demonstrated (Figures 4A–C), expression of ANF was increased to the level of control (fold difference flox/flox/Cre vs. flox/flox, means ± S.E., 1.11 ± 0.02, n=2), however the expression of BNP remained reduced (0.2 7 ± 0.00, n=2) and that of βMHC remained upregulated (5.12 ± 0.39, n=2).

In hearts with perinatal loss of Nkx2–5, marked reduction (80–90%) of three additional gene products important for cardiac conduction and contraction, including Na^+ channel $Na_v1.5(\alpha)$, ryanodine receptor 2 and cardiac MLCK, was also demonstrated by PD12.15,16 Reduction in expression was also observed in hearts from mice with loss of Nkx2–5

beginning at 2 weeks of age (Figure 5C); however the magnitude of the reduction was less and the time-course was prolonged in hearts with Nkx2–5 knockout from 2 weeks of age compared to perinatal loss of Nkx2–5. Taken together, a slower progression and decreased magnitude of reduction of Nkx2–5 downstream targets characterize the phenotype of hearts with loss of Nkx2–5 from 2 weeks of age. This observation is one potential mechanism explaining the slower disease progression.

Discussion

Nkx2–5 is a critical factor in fetal cardiac development when cardiomyocytes are proliferating as demonstrated in various studies using mouse embryos or stem cells.13,14 Nkx2–5 is also important in perinatal hearts, as demonstrated by rapid conduction and contraction defects observed within 4 days after deletion of Nkx2–5 following tamoxifen injection, leading to premature death.15 Loss of Nkx2–5 from 2 weeks of age results in an overall similar phenotype, but with a distinct disease progression and severity despite using a genetically identical mouse model with 2 weeks difference in tamoxifen-injection. For example, perinatal loss of Nkx2–5 results in premature death, but loss of Nkx2–5 from 2 weeks of age does not. Apparent phenotype emerges within 4 days with perinatal loss of Nkx2–5 vs. over 7 weeks with loss beginning at 2 weeks of age.

With respect to the pathogenesis of human congenital AV block and occasional LV dysfunction associated with NKX2–5 mutations, our previous15 and current studies reveal that Nkx2–5 is necessary for proper conduction and contraction postnatally, but is more critical in the perinatal heart and is necessary for survival in mice. Haploinsufficiency (loss of one allele) of Nkx2–5 is considered an underlying disease mechanism of human congenital heart disease as demonstrated in several germline heterozygous-null Nkx2–5 mice.26–29 While substantially different degrees of defects among studies were reported, PR prolongation accompanied with wide QRS were detected as early as 7 weeks of age.28 When the Nkx2–5 gene was deleted after 2 weeks of age, heterozygous-null mice did not apparently develop heart enlargement, PR-prolongation or wide QRS by 24 weeks of age, with exception of one mouse with intermittent 2° AV block at 24 weeks of age.

We initiated this study based upon the hypothesis that cardiomyocytes before and after G1 cell-cycle arrest may demonstrate the morphological, metabolic and gene expression changes with reorganization of nuclear architecture and chromatin structure.19,20 In fact, we found a global increase of DNA methylation in hearts within 2 weeks after birth using 5-methylcytosine staining (data not shown).30 The question remains as to how an increase of broad DNA methylation relates to slower and milder phenotypes in mice with loss of Nkx2–5 from 2 weeks age. It might be argued that a compact chromatin DNA structure due to DNA methylation may delay transcriptional re-regulation after loss of Nkx2–5 from 2 weeks of age. Another mechanism is that other transcription factors might compensate for the absence of Nkx2–5 after 2 weeks of age. The mRNA half-life of Nkx2–5 targets after 2 weeks of age might be substantially longer compared to at the neonatal stage.

To our knowledge, a limited number of cardiac transcription factors have been studied using postnatal-specific gene deletion. Loss of serum responsive factor (SRF) from 2 months of

age leads to lethal heart failure 10 weeks after tamoxifen injection.31 Although a 70% reduction of SRF downstream target, α-actin, was demonstrated 5 days after tamoxifen injections, its expression did not change appreciably 30 and 60 days after tamoxifen injection. Expression of other SRF targets, such as vinculin and zyxin, demonstrated in ES cells, as well as Nkx2–5, MEF2C, GATA4 and TEF1 demonstrated following loss of SRF in embryonic hearts (floxed-SRF mice crossed with βMHC-Cre transgenic mice), was unchanged or fluctuated 5, 30 and 60 days after tamoxifen injection.31 Thus, this study also demonstrated differential regulation of transcription in adult vs. proliferating cardiomyocytes. Additional studies using postnatal-specific gene targeting of cardiac transcription factors will reveal whether the developmental stage-dependent effects are a general property of cardiac transcription factors.

Nkx2–5 has been shown to be involved in cardiac hypertrophy;32–34 however the present study shows that without Nkx2–5, ventricular cardiomyocytes have compensatory hypertrophy at 24 weeks of age. Thus, Nkx2–5 may not be critically important for adult ventricular cardiomyocyte hypertrophy as was suggested in a recent review.35 In contrast, Nkx2–5 might be important for cellular enlargement in AV nodal cardiomyocytes during postnatal development as demonstrated in this study, which are not involved in contraction nor are they affected by compensatory hypertrophic stimuli. Of note, defects in AV bundle development during embryonic stages have been demonstrated in heterozygous Nkx2–5 knockout mice.29 Morphological changes in ventricular conduction systems including AV bundle remain to be analyzed in postnatal Nkx2–5 knockout hearts.

Cardiomyocyte hypertrophy is usually accompanied with increase in expression of ANF and BNP. The expression of those markers in the hypertrophic cardiomyocytes with loss their upstream regulatory transcription factor, Nkx2–5, is somewhat difficult to interpret and is interesting. We found that early after loss of Nkx2–5 (3.5 and 6 weeks of age, Figure 5B), expression of ANF and BNP was decreased due to loss of their transactivator, Nkx2–5. At 24 weeks of age when cardiomyocyte hypertrophy was evident, ANF expression was increased to the normal level, but BNP expression remained downregulated. One potential interpretation of time-dependent changes in ANF expression (downregulation early after loss of Nkx2–5 and normalization at 24 weeks of age) would be due to some Nkx2–5-independent regulatory mechanism at 24 weeks of age, which are highly induced and overcome loss of Nkx2–5 function in transcription of ANF gene, resulted in increase of ANF expression to the normal control level.

In summary, we demonstrated that Nkx2–5 actively regulates a critical set of genes in postnatal cardiomyocytes to maintain proper cardiac function. However an age-related phenotypic difference after loss of Nkx2–5 between 2 weeks of age vs. perinatal stage was identified, which is accompanied by a slower and smaller reduction of several critical Nkx2–5 responsive genes.

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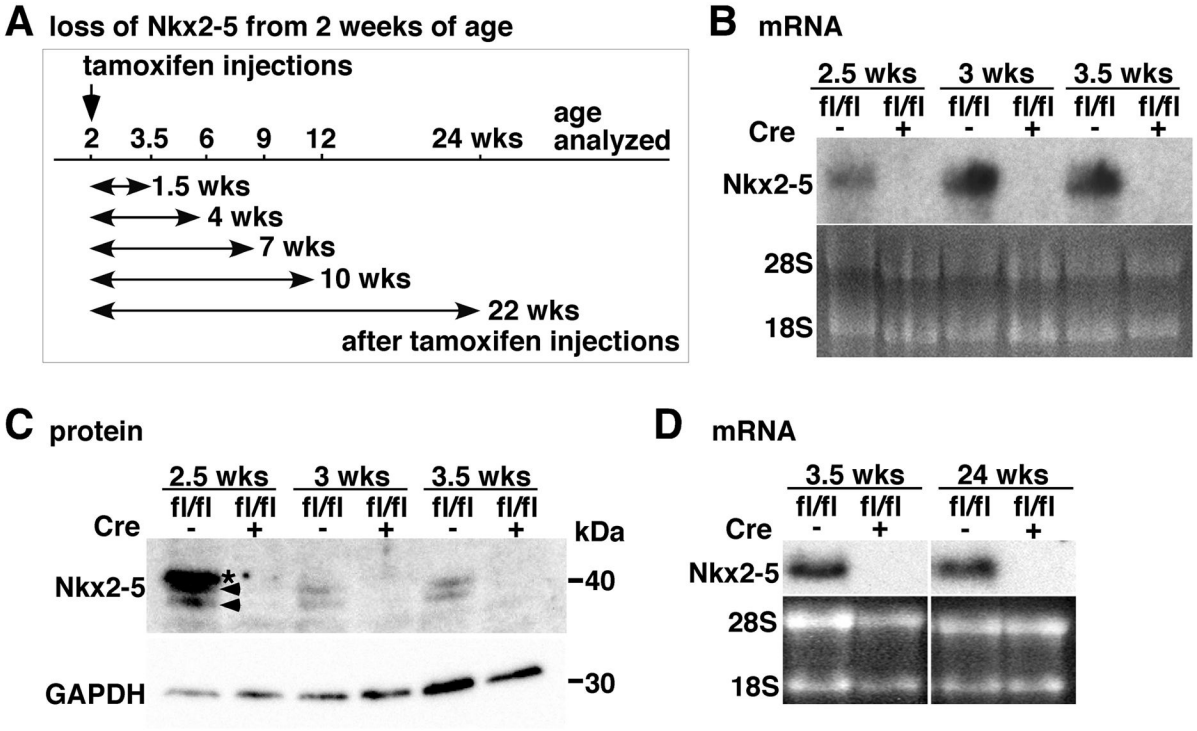
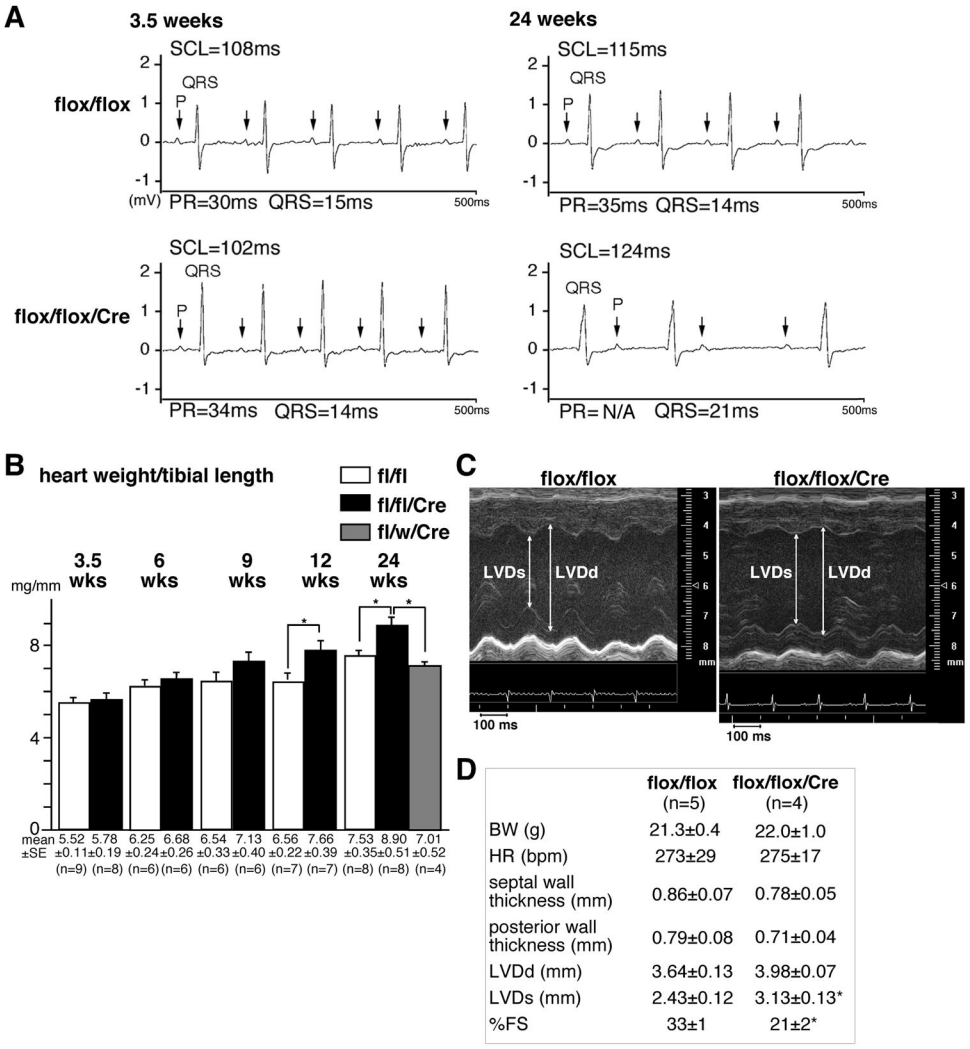


Figure 1. Time course study of tamoxifen-inducible targeting of Nkx2–5 beginning at 2 weeks of age

(A) Experimental time-course of Nkx2–5 knockout beginning at 2 weeks of age. (**B**) Northern blotting demonstrated that nearly complete loss of Nkx2–5 mRNA from 2.5 weeks (immediate after completion of four consecutive tamoxifen injections) to 3.5 weeks in flox/flox/Cre compared to flox/flox mice. (**C**) Western blotting demonstrated marked reduction of Nkx2–5 proteins (arrowheads) from 2.5 to 3.5 weeks of age in flox/flox/Cre mice. Non-specific band was detected at 2.5 weeks of age (marked with*). (**D**) Northern blotting demonstrated reduced expression of Nkx2–5 in flox/flox/Cre compared to flox/flox mice at 3.5 and 24 weeks of age after tamoxifen injections. fl, flox; wks, weeks.



	flox/flox (n=5)	flox/flox/Cre (n=4)
BW (g)	21.3±0.4	22.0±1.0
HR (bpm)	273±29	275±17
septal wall thickness (mm)	0.86±0.07	0.78±0.05
posterior wall thickness (mm)	0.79±0.08	0.71±0.04
LVDd (mm)	3.64±0.13	3.98±0.07
LVDs (mm)	2.43±0.12	3.13±0.13*
%FS	33±1	21±2*

Figure 2. AV block and heart enlargement in Nkx2–5 knockout mice

(A) Examples of telemetry ECG recording at 3.5 and 24 weeks of age. At 24 weeks of age, telemetry ECG recording from flox/flox mouse showed normal sinus rhythm (left panel), however from flox/flox/Cre showed 2° AV block (right panel). See Table 1. (**B**) Heart weight/tibial length in Nkx2–5 knockout mice compared to control mice (flox/flox or flox/wild/Cre), with significant increase in heart weight/tibial length in Nkx2–5 knockout mice at 12 and 24 weeks of age. (**C, D**) Representative imaging of M-mode ultrasound microscope and echocardiographic indices of control and Nkx2–5 knockout mice approximately 24 weeks of age (mean ± S.E.) are shown. SCL, sinus cycle length (beta-to beat heart rate); BW, body weight; HR, heart rate; LVDd, left ventricular diastolic dimension; LVDs, left ventricular systolic dimension; %FS, percentage of left ventricular fractional shortening.

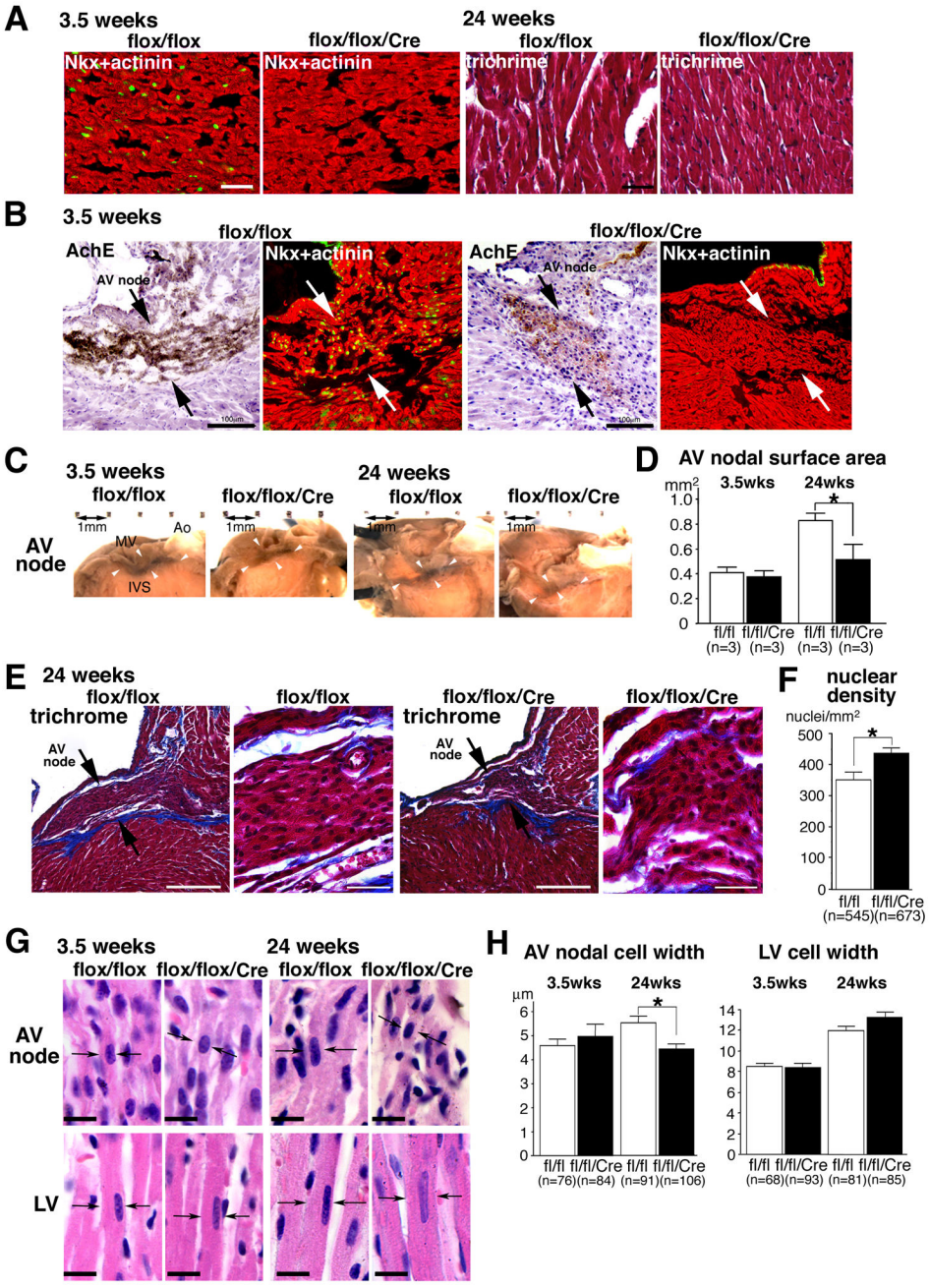


Figure 3. Nkx2–5 protein expression in contractile myocardium and AV node

(A) Co-immunostaining of Nkx2–5 and sarcomeric actinin demonstrate reduced expression of Nkx2–5 protein in ventricles dissected from Nkx2–5 mice at 3.5 weeks of age (left panels). Heart sections from 24 weeks old mice stained with Masson's trichrome show no apparent interstitial fibrosis in Nkx2–5 knockout mice compared to control mice (right panels). Bars = 100 μm. (**B**) Tissue sections of 3.5 week old mouse heart demonstrates that AV node is positive for acetylcholine esterase staining (AchE) both in flox/flox and flox/flox/Cre mice. The adjacent tissue sections including AV node stained positive with anti-Nkx2–5 antibody (green) in flox/flox mice but was negative in flox/flox/Cre mice.

Sarcomeric actinin staining is shown in red. Bars = 100 μm. (**C**) Whole mount acetylcholine esterase staining demonstrates AV node (brown staining, arrowheads) both in flox/flox and flox/flox/Cre mice at 3.5 and 24 weeks of age. Scale bars = 1 mm. (**D**) Surface area positive for acetylcholine esterase compared between flox/flox and flox/flox/Cre mice at 3.5 and 24 weeks of age (mean ± S.E.). *$p < 0.05$. (**E**) No apparent fibrosis was observed in Nkx2–5 knockout AV node analyzed by Masson's trichrome staining at 24 weeks of age. AV nodal area is marked with arrows. Bars in low magnifications = 200 μm. Bars in high magnifications = 50 μm. (**F**) Nuclear density (number of nuclei/mm^2) in flox/flox and flox/flox/Cre mice at 24 weeks of age. Total nuclear number counted was from a total of 3 different flox/flox or flox/flox/Cre mice. (**G**) HE-stained longitudinal sections of cardiomyocytes in AV node (left upper panels) and LV upper free wall (left bottom panels) were utilized for measurement of cell width at the nuclei level at 3.5 and 24 weeks of age. Bars = 20 μm. (**H**) AV nodal cell width (left panel) and LV cell width (right panel) compared between flox/flox and flox/flox/Cre mice at 3.5 and 24 weeks of age (mean ± S.E.). *$p < 0.05$. Total cell number counted was from a total of 3 different flox/flox or flox/flox/Cre mice at 3.5 weeks and 4 different flox/flox or flox/flox/Cre mice at 24 weeks. MV, mitral valve; Ao, aorta; IVS, interventricular septum.

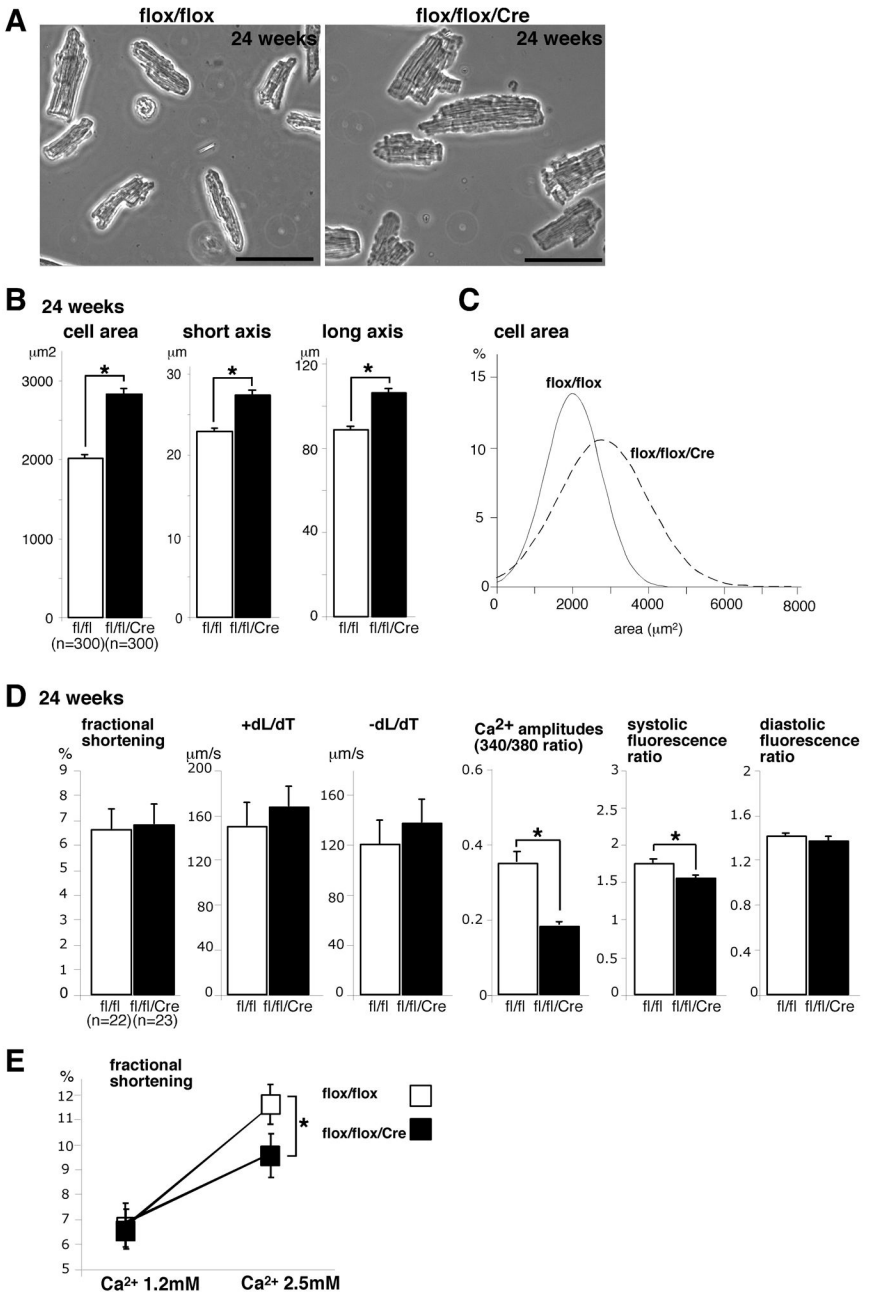


Figure 4. Increased cell size and Ca^{2+} handling defects in cardiomyocytes isolated from Nkx2–5 knockout heart

(A) Representative images of cardiomyocytes isolated from flox/flox (left) and flox/flox/Cre mice (right) at 24 weeks of age. Increased cell size was demonstrated in flox/flox/Cre mice. Bars = 100 μm. (**B**) Cell area (μm^2), short axis (μm) and long axis (μm) of cardiomyocytes isolated from flox/flox or flox/flox/Cre mice (n=300 from 3 mice). (**C**) Distribution of the cell area of cardiomyocytes isolated from flox/flox and flox/flox/Cre mice. (**D**) Measurements of cardiac contraction and simultaneous Ca^{2+} transients in isolated cardiomyocytes from control flox/flox (white bars) or Nkx2–5 knockout (flox/flox/Cre)

mice (black bars). Summarized data (mean ± S.E.) demonstrate that Nkx2–5 knockout cardiomyocytes show decreased Ca^{2+} amplitude, systolic fluorescence ratio, but preserved %fractional shortening, +dL/dT, −dL/dT and diastolic fluorescence ratio. *$p < 0.05$. (**E**) Increase of Ca^{2+} concentration in superfusate from 1.2 to 2.5 mM, increased %fractional shortening both in control and Nkx2–5 knockout cardiomyocytes, but was less in Nkx2–5 knockout cardiomyocytes compared to control cardiomyocytes.

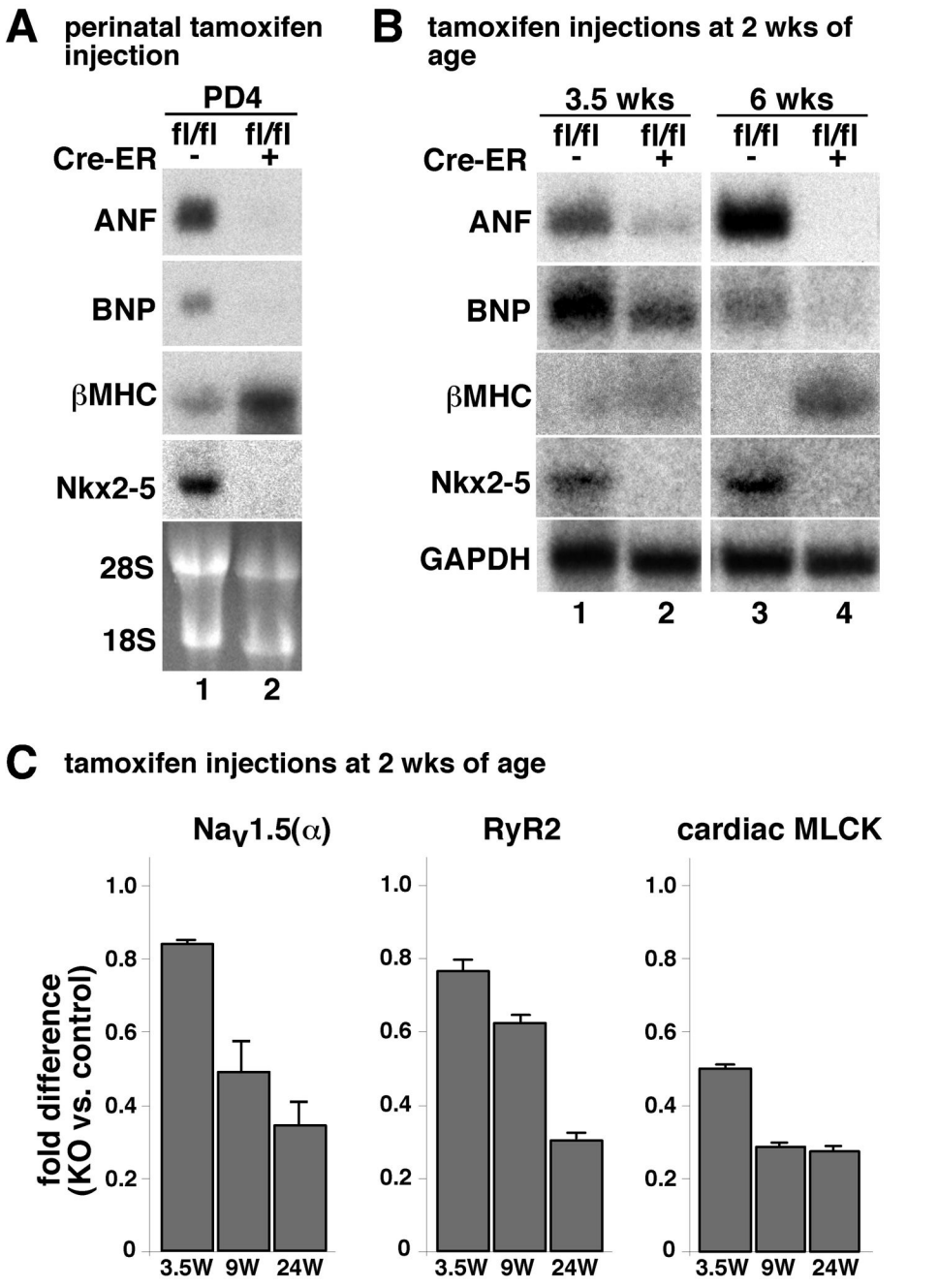


Figure 5. Expression of Nkx2–5 target genes in Nkx2–5 knockout hearts

(A) Northern blotting shows reduction of ANF and BNP mRNA and induction of βMHC mRNA in perinatal Nkx2–5 knockout hearts at PD4. (**B**) Northern blotting shows reduction of ANF and BNP mRNA at 3.5 and 6 weeks of age and induction of βMHC mRNA at 6 weeks of age in Nkx2–5 knockout hearts when tamoxifen was injected from 2 weeks of age. (**C**) Real-time RT-PCR shows fold difference (Nkx2–5 knockout vs. control) of mRNA of cardiac MLCK, $Na_V1.5(\alpha)$ and RyR2 at 3.5 weeks, 9 weeks and 6 month of age. MLCK, myosin light chain kinase; RyR2, ryanodine receptor 2; KO, knockout.

Table 1

Electrocardiographic data of Nkx2–5 deletion from 2 weeks of age (telemetry)

		SCL (ms)	PR (ms)	HR (bpm)	QRS (ms)	QT (ms)	QTc (ms)	Intermittent 2° AV block
3.5 wks	fl/fl (n=4)	98±2	36±1	613±12	15±1	45±1	45±1	0/4
	fl/fl/Cre (n=5)	99±8	40±3	622±51	14±1	44±2	45±1	0/5
6 wks	fl/fl (n=5)	88±3	37±2	687±22	14±1	38±4	41±4	0/5
	fl/fl/Cre (n=5)	91±4	40±1	664±27	14±1	38±5	39±4	1/5
9 wks	fl/fl (n=6)	97±3	37±1	618±18	17±1	46±1	47±1	0/6
	fl/fl/Cre (n=6)	96±5	47±3*	631±29	15±1	39±4	39±3	5/6
	fl/w/Cre (n=5)	92±4	36±1	653±25	15±1	43±2	45±1	0/5
12 wks	fl/fl (n=6)	95±3	37±1	633±18	16±1	45±2	46±1	0/6
	fl/fl/Cre (n=6)	110±4*	49±3*	548±20*	18±1	46±4	44±4	6/6
	fl/w/Cre (n=5)	89±2	36±1	677±16	15±1	44±1	46±2	0/5
24 wks	fl/fl (n=6)	97±3	40±1	620±18	16±1	46±1	46±1	0/6
	fl/fl/Cre (n=6)	100±3	52±3*	602±18	21±1*	46±3	46±3	6/6
	fl/w/Cre (n=4)	106±6	39±2	574±31	16±2	44±4	43±3	1/4

Values are as mean ± S.E.

*$p<0.05$. SCL, sinus cycle length; HR, heart rate (beat per minute); QTc, rate-corrected QT interval; wks, weeks; fl, flox; w, wild.

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