Informatic and Functional Approaches to Identifying a Regulatory Region for the Cardiac Sodium Channel

Thomas C. Atack, Dina Myers Stroud, Hiroshi Watanabe, Tao Yang, Lynn Hall, Susan B. Hipkens, John S. Lowe, Brenda Leake, Mark A. Magnuson, Ping Yang, Dan M. Roden

Rationale: Although multiple lines of evidence suggest that variable expression of the cardiac sodium channel gene SCN5A plays a role in susceptibility to arrhythmia, little is known about its transcriptional regulation. Objective: We used in silico and in vitro experiments to identify possible noncoding sequences important for transcriptional regulation of SCN5A. The results were extended to mice in which a putative regulatory region was deleted.

Methods and Results: We identified 92 noncoding regions highly conserved (>70%) between human and mouse SCN5A orthologs. Three conserved noncoding sequences (CNS) showed significant (>5-fold) activity in luciferase assays. Further in vitro studies indicated one, CNS28 in intron 1, as a potential regulatory region. Using recombinase-mediated cassette exchange (RMCE), we generated mice in which a 435-base pair region encompassing CNS28 was removed. Animals homozygous for the deletion showed significant increases in SCN5A transcripts, NaN1.5 protein abundance, and sodium current measured in isolated ventricular myocytes. ECGs revealed a significantly shorter QRS (10.7±0.2 ms in controls versus 9.7±0.2 ms in knockouts), indicating more rapid ventricular conduction. In vitro analysis of CNS28 identified a short 3′ segment within this region required for regulatory activity and including an E-box motif. Deletion of this segment reduced reporter activity to 3.6±0.3% of baseline in CHO cells and 16%±3% in myocytes (both P<0.05), and mutation of individual sites in the E-box restored activity to 62%±4% and 57%±2% of baseline in CHO cells and myocytes, respectively (both P<0.05).

Conclusions: These findings establish that regulation of cardiac sodium channel expression modulates channel function in vivo, and identify a noncoding region underlying this regulation. (Circ Res. 2011;109:38-46.)

Key Words: gene expression regulation ▪ sodium channels ▪ mice ▪ transgenic

Normal function of the sodium channel encoded by SCN5A is critical to initiation of the action potential and its propagation in atrium and ventricle.1 Mutations that decrease sodium current (INa) by disrupting channel processing or function cause a series of overlapping human arrhythmia syndromes, including Brugada Syndrome and conduction system disease.1,2 In subjects of Asian ancestry, we have described a common variant in the SCN5A core promoter that modulates the duration of the QRS interval, an index of ventricular conduction, in normal subjects.3 In addition, the promoter variant appeared to modulate the extent to which drug challenge prolonged QRS in patients with the Brugada Syndrome. Notably, QRS prolongation is a hallmark of sodium channel block by drugs, and sodium channel blockers are well recognized to have proarrhythmic potential.4 Taken together, these findings implicate variability in SCN5A expression as a mechanism underlying arrhythmia susceptibility in the whole heart. To date, few studies have addressed mechanisms underlying transcriptional control of SCN5A expression. We have previously identified the core promoter of human SCN5A and common polymorphisms in that region.5,6 Others have reported that transgenic cardiac expression of the putative repressor Snail led to decreased INa and dilated cardiomyopathy; further experiments suggested that Scn5a is a Snail target.7 Snail is zinc-finger transcription factor known to target E-box motifs.8 Shang and Dudley reported multiple alternate 5′-splice variants of the murine sodium channel ortholog; these were developmentally regulated, and both enhancer and repressor regulatory elements and an alternate promoter were identified.9

Original received October 28, 2010; revision received April 8, 2011; accepted May 4, 2011. In April 2011, the average time from submission to first decision for all original research papers submitted to Circulation Research was 15 days.

From the Department of Medicine (T.C.A., D.M.S., H.W., T.Y., L.H., J.S.L., B.L., P.Y.), Molecular Physiology (S.B.H., M.A.M.), and Biophysics (S.B.H., M.A.M.) and the Department of Pharmacology (D.M.R.), Vanderbilt University School of Medicine, Nashville, Tennessee.

Hiroshi Watanabe is currently affiliated with the Division of Cardiology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan.

Correspondence to Dan M. Roden, MD, Director, Oates Institute for Experimental Therapeutics, Assistant Vice-Chancellor for Personalized Medicine, Vanderbilt University School of Medicine, 2215B Garland Avenue, 1285 MRBIV Light Hall, Nashville, TN 37232-0575. E-mail dan.roden@vanderbilt.edu

© 2011 American Heart Association, Inc.

Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/CIRCRESAHA.110.235630
In this report, we first identified short sequences highly conserved between mouse and human. Further studies implicated one of these conserved noncoding sequences (CNS), designated CNS28 and located 1.3 kb upstream of exon 2, as a potential regulator of channel expression. To further test this hypothesis in vivo, we determined the electrophysiological properties of mice in which the CNS28 region was deleted. We find that the absence of CNS28 results in striking increases in sodium channel expression in the intact heart, with attendant increased sodium current and conduction. Additional experiments in heterologous cells and cardiomyocytes implicate the loss of an E-box binding site as responsible for this increase in sodium channel expression.

**Methods**
Details for each method are presented in the Online Supplement available at http://circres.ahajournals.org.

**Identification of Potential Regulatory Regions**
To identify CNS elements, we compared the human SCN5A locus with its mouse ortholog using the VISTA Genome Browser (http://pipeline.lbl.gov/cgi-bin/gateway2). Each of 92 CNS elements identified was then PCR amplified and assayed for activity as described below and in the On-Line Supplement. Those showing >5-fold increase in reporter activity in luciferase assays were then analyzed for potential muscle-specific transcriptional regulatory modules using the M-SCAN algorithm (http://www.cisreg.ca/cgi-bin/mscan/MSCAN). For identification of potential repressive transcription factors in CNS28, we used rVista (http://rvista.dcode.org/) to compare human and mouse sequences for conserved transcription factor–binding sites.

**Reporter Constructs**
Reporter constructs measuring the activity of all 92 CNS constructs (Online Table I), human CNS28 with the full-length human SCN5A promoter (Table), and deletion analysis of the alternate mouse Scn5a promoter (Table) were generated by cloning PCR fragments into the pGL3-promoter or pGL3-basic vectors (Promega). Mutagenesis of the DC3 deletion fragment was performed using the QuickChange XL II kit (Stratagene) using the primers listed in Table. Reporter assays were conducted as previously described in CHO cells and in cardiomyocytes isolated from 1- to 2-day-old mice.

**Generation of CNS28−/− Mice**
We used mouse embryonic stem (ES) cells in which a region of the Scn5a gene was modified to allow recombinase-mediated cassette exchange (RMCE) to be used to easily generate animals containing allelic variants of human sodium channels under control of SCN5A regulatory sequences. Using this approach, we previously generated H/H mice in which the targeted region was replaced by the human SCN5A full-length cDNA. For the present experiments, we modified the original H exchange construct to delete bases 1720 to 2154 (that encompass the CNS28 region), and then performed RMCE as previously described to generate CNS28−/− animals. After removal of the hygromycin resistance cassette by breeding to a Flpe-expressing line, mice were then interbred to generate CNS28−/− animals. Experiments began after 3 backcrosses using littermate H/H mice as controls. All experiments performed on mice were approved by the institutional animal care and use committee.

**Quantitative Real-Time RT-PCR**
Total mRNA from adult mice atria or ventricles was isolated using the TRizol method (Invitrogen) and cDNA was prepared using the Transcripter First-Strand cDNA Synthesis kit (Roche). Quantitative real-time RT-PCR (qPCR) on SCN5A was performed with TaqMan probes targeting human SCN5A (Hs00156939_m1) using beta actin (Mm00607939_s1) as a reference gene. The qPCR targeting mouse Scn1b was performed using TaqMan probe (Mm00441210_m1) with Hprt1 (Mm00446968_m1) as the reference gene. Analysis was performed using SDS 2.2 software (Applied Biosystems).

**Western Blotting**
Western blotting protocol and semiquantitative protein analysis are described in the Online Supplement. ECG (ECG) and Lido Challenge ECGs and drug challenges were recorded as previously described.

**Isolation of Mouse Ventricular Cardiomyocytes and Sodium Current Recordings**
Adult H/H and CNS28−/− mouse cardiac ventricular myocytes were isolated by a modified collagenase/protease method, and sodium current studied as previously described. Protocols and data analysis are presented in the Online Supplement.

**Echocardiogram**
Transthoracic echocardiograms were performed on resting conscious mice at the murine cardiovascular core, Vanderbilt University, as previously described. Signals were acquired using a 15-MHz transducer (Sonos 5500 system, Agilent) and analyzed by a sonographer who was blind to the genotype.
Data Analysis

Results are presented as mean±SE, and statistical comparisons were made using the unpaired Student t test. A value of *P*<0.05 was considered statistically significant.

Results

Conserved Nucleotide Sequences

In order to identify genomic elements that may play a role in the transcriptional regulation of SCN5A, we compared human and mouse SCN5A sequences to identify conserved regions. Using the genomic sequence comparison program VISTA, we identified 92 CNS elements 77 to 446 base pairs (bp) long with >70% nucleotide identity between the human locus and its mouse ortholog. We identified 12 out of 92 CNS elements in the 47.7-kb 5′ upstream region between SCN5A exon 1 and the terminal exon of SCN10A gene, which is just upstream, and 15 of 92 CNS elements were identified in the 26.4-kb 3′ downstream region between the terminal exon (exon 28) of SCN5A and the exon 1 of the downstream gene ENDOG1L. The highest density was in intron 1, where 23 CNS elements were contained in 16 kb (Figure 1A). Ten introns contained no CNS elements.

We tested the effect of each CNS on the SV40 promoter driving luciferase both in Chinese hamster ovary (CHO) cells and cardiomyocytes. There were 3 elements, CNS23, CNS28, and CNS32, that demonstrated a >5-fold increase in reporter activity (Figure 1B). The MSCAN analysis, which we used to identify potential functionally important clusters of muscle-specific transcription factors, designated 3 tandem TEF-1 (transcriptional enhancer factor 1) sites and an SRF (serum response factor) recognition site in CNS28 (Figure 1C), and no elements in the other 2. There was a high degree of

Figure 1. Identification of CNS28. A, A portion of the VISTA human-to-mouse sequence comparison for SCN5A with the locations of Exon 1, Exon 2, and CNS28 marked. The peaks show regions of high conservation across species. Peaks that are shaded satisfy the CNS selection criteria of 70% identity between sequences. B, Initial luciferase experiments of all 92 CNS constructs in CHO cells and cardiomyocytes with CNS23, CNS28, and CNS32 marked by arrows. C, Human CNS28 aligned with mouse, dog, cow, and pig orthologs with the TEF-1 and SRF sites suggested by MSCAN marked.
conservation across species at the SRF and at one of the TEF-1 sites. Accordingly, our further studies focused on the activity of CNS28, a 435-bp region located in the 5′ portion of intron 1.

Generating CNS28−/− Mice

In our previous studies, we replaced a region flanking the endogenous exon 2 transcription site with a construct that contained approximately 2 kb of mouse intron 1 along with the human SCN5A cDNA. In homozygous mice, designated H/H, no murine Scn5a expression was detected; ventricular myocyte sodium current and electrocardiograms (ECGs) were identical to those in wild-type animals, indicating that the human channel generated physiological channel function. In the present experiments, we used site-directed mutagenesis to remove the CNS28 element from our original exchange construct (Figure 2A) and then used RMCE to generate CNS28−/− mice. Loss of CNS28 was confirmed by

Figure 3. Relative transcript and protein amounts in H/H and CNS28−/−. SCN5A atrial mRNA (A) and ventricular mRNA (B) levels analyzed by quantitative real-time PCR. CNS28−/− mice had 42%±12% more expression in atria and 71%±23% more in ventricles than did H/H. C, Whole-heart protein expression levels analyzed by Western blot. Bands were analyzed on the same gel. D, Band densitometry analysis of the Western blots showing relative Na+/K+ abundance in heart was 31%±10% higher in CNS28−/− mice and 67%±23% higher in CNS28−/− mice. Atack et al Sodium Channel Regulator 41
genotyping (Figure 2B), and matings of CNS28+/− × CNS28+/− mice yielded pup distributions in Hardy Weinberg equilibrium with 32 H/H, 64 CNS28+/−/H11002, and 41 CNS28+/H11002/H11002.

Sodium Channel Expression in CNS28+/− Mice

Our initial results indicated that the CNS28 fragment increased SV40-mediated reporter activity. However, in CNS28+/−/H11002 mouse hearts, quantitative real-time PCR (qPCR) showed that SCN5A transcripts levels were 42%±12% more abundant in atria and 71%±23% more abundant in ventricles than in H/H mice (P<0.05 in both tissues, Figure 3A and 3B). Western blotting showed concordant results: calnexin-normalized Na1.5 abundance in heart was 31%±10% higher in CNS28+/− and 67%±23% higher in CNS28+/−/H11002 mice than in H/H mice (P<0.05 for both CNS28+/− and CNS28+/−/H11002 when compared with H/H, Figure 3C and 3D).

These changes translated into increases in functional sodium channels (Figure 4A and 4B). Peak I_{Na} amplitude measured at −30 mV was 59%±14% greater in CNS28+/−/H11002 ventricular myocytes in comparison with that in H/H cells (P<0.05, Figure 4C). Interestingly, we also observed small but significant negative shifts in the voltage dependence of sodium channel activation and inactivation in CNS28+/−/H11002 mice (Figure 4D).

CNS28+/−/H11002 mice also showed ECG changes consistent with these findings. In comparison with WT, homozygotes displayed 12.3% shorter PR intervals (38.8±0.6 ms versus 34.1±0.7 ms), 9.3% shorter QRS intervals (10.7±0.2 ms versus 9.7±0.2 ms), and 7.0% shorter QT intervals (52.9±0.8 ms versus 49.2±1.2 ms) (all P<0.05, Figure 4E). After challenge with the sodium channel blocker lidocaine, these intervals increased to a similar absolute extent in both genotypes, prolonging PR by 14.1 and 13.9 ms and QRS by 4.1 and 3.2 ms in H/H and CNS28+/−/H11002, respectively. Flecainide challenge showed similar results (data not shown).

In Vitro Transcriptional Regulation by CNS28

The finding that deleting CNS28 increased channel transcripts and protein, with functional consequences, indicates that this region includes sequences that suppress sodium channel expression in vivo. This result is at odds with the initial screening experiment that identified CNS28 as a potential positive regulatory sequence.
On the basis of these results we assessed reporter activity of constructs in which human CNS28 was included with the full-length *SCN5A* promoter rather than the SV40 promoter used in the initial experiments. We compared activity of 2 constructs, each containing the *SCN5A* promoter and luciferase, 1 with and 1 without CNS28 (Figure 5A). The construct without CNS28 increased luciferase expression 33%±4% in CHO cells and 87%±35% in myocytes (Figure 5B and 5C, both *P*<0.05), consistent with the repressor function indicated by the in vivo findings.

We have previously identified a region upstream of exon 1 as the core *SCN5A* promoter.5,6 Experiments conducted by Shang and Dudley revealed a second, 1363-bp *Scn5a* promoter in the mouse.9 The 5′ 435 bp of this promoter are what we identified in our screen as CNS28. Their analysis suggested that the 5′ 480 bp contained a repressive element.9 We hypothesized that CNS28 contained this repressive activity and generated a series of smaller deletions of the alternative promoter that they described (Figure 6A). The rVista analysis identified 30 possible transcription factor–binding sites conserved between the human and mouse, and therefore break points were chosen between clusters of these sites (Online Figure I). The full alternative promoter–luciferase construct (1,361 bp) was designated deletion construct 0 (DC0), and contains all the features of the Shang and Dudley promoter stated above. Deletion construct 1 (DC1) removes the initial 50 bp and deletion construct 2 (DC2) an additional 100 bp. Deletion construct 3 (DC3) eliminates 285 bp of the alternate promoter. Deletion construct 4 (DC4) takes away the entirety of CNS28 from the alternate promoter by deleting the first 435 bp.

In our luciferase reporter assay, the activity of DC0 was used as the baseline reference in both CHO cells and myocytes. Constructs DC1 and DC2 showed no difference in activity versus DC0 in either cell type. The DC3 construct virtually abolished reporter activity in both cell types, reducing activity to 3.3%±0.1% of DC0 in CHO cells, and 12%±3% in myocytes (both *P*<0.05). The last construct, DC4, restored activity to 79%±6% and 67%±11% of DC0 in CHO cells and myocytes, respectively (Figure 6B and 6C, both *P*<0.05). There are 2 points illustrated by these data. First, between DC2 and DC3 there is likely an enhancer element that can counter the negative transcriptional activity we observe with CNS28; therefore, DC3 shows the highest repressive activity. Second, removal of 150 base pairs between DC3 and DC4 results in loss of that repressive activity. Sequence analysis of this region shows that it contains an E-box motif (consensus sequence CANNTG).23 The repressor Snail has previously been shown to bind E-boxes in the core promoter of *SCN5A*. Therefore we tested the hypothesis that this E-box may be important for repressor binding. When the motif was mutated in DC3 (CATATG to CTTAAG), activity was restored to 62%±4% of the activity of DC0 in CHO cells and 66%±10% in myocytes (Figure 6D and 6E, both *P*<0.05). This increase in activity is similar to the increase we see between DC3 and DC4, in which the final segment containing the E-box is removed, further supporting our hypothesis.

**Figure 5. Activity of human CNS28 on the full-length human *SCN5A* promoter.** A, Graphical representation of the vectors used. Luciferase activity driven by the full-length human *SCN5A* promoter with and without CNS28 in CHO cells (B) and neonatal cardiomyocytes (C). *SCN5A* promoter with CNS28 is designated 100% activity. When CNS28 is not present, luciferase activity increases by 33%±4% in CHO cells and 87%±35% in myocytes. *P*<0.05.
Sodium Channels Are Required for Normal Cardiac Function

Na\textsubscript{1.5} expression is absolutely required for normal cardiac function; knockouts in mice and in fish are embryolethal and cause cardiac developmental abnormalities.\textsuperscript{24,25} More modest reduction of sodium current slows cardiac conduction and creates an arrhythmia-prone heart in the setting of monogenic disease\textsuperscript{26,27} or during therapy with sodium channel–blocking drugs.\textsuperscript{4} We have previously found an association between a variant haplotype in a regulatory region of the cardiac sodium channel promoter commonly observed in Asian subjects and variable QRS duration, and recent genome-wide association studies have identified an association between the SCN5A-10A locus and variable PR and QRS durations.\textsuperscript{28–30} These data support the general hypothesis\textsuperscript{31,32} that variable cardiac ion channel transcription modulates the electrophysiological properties of the heart. A contrary view is that feedback mechanisms in the mammalian heart regulate transcription to achieve a tight range of normal electrophysiological behaviors,\textsuperscript{32} although monogenic diseases producing striking electrocardiographic changes argue against such tight control. Previous work in this area has focused on in vitro approaches and on establishing the functional consequences of coding region variants in genetically modified mice. However, no study to date has directly evaluated the functional consequences of deleting noncoding potential regulatory regions of a cardiac ion channel gene.

CNS28 Regulates Cardiac Sodium Channel Expression

Our initial in silico screen identified 92 highly conserved noncoding sequences, and preliminary reporter experiments, using the SV40 promoter, suggested that one, CNS28, included positive regulatory elements for SCN5A. Driven by this initial result, we generated CNS28/ mice, and unexpectedly, these animals demonstrated dramatic increases in sodium channel transcripts and channel protein expression. This increased Na\textsubscript{1.5} is functional: the CNS28/ mice have significantly larger peak \( I_{\text{Na}} \) in isolated ventricular myocytes and shorter PR and QRS, indicating rapid ventricular conduction. The increased sodium current also displayed unexpected shifts in the voltage dependence of channel activation and inactivation; the mechanism requires further study, but one possibility is a change in the extent of interaction with ancillary proteins, such as \( \beta \)-subunits, that are known to modulate channel gating.\textsuperscript{33} For example, using real-time PCR, we found no alterations in expression of Scn1b in CNS28/ mice (Online Figure II). This supports the notion that although more sodium channels are present, concomitant increases in function modifying protein partners may not occur. Changes in the Na\textsubscript{1.5} macromolecular complex could lead to alteration in channel gating. Further experiments examining changes in expression and localization of other Na\textsubscript{1.5} partners\textsuperscript{34} may assist in elucidating the underlying mechanism. Another possibility is raised by the clustering of Scn5a with Scn10a and 11a in the mouse and...
human genomes; thus, it is conceivable that removal of CNS28 impacts the level of expression of these channels in the heart. Such a change in the channel profile in the heart could result in shifts in the inactivation and activation of cardiac sodium current. However, in preliminary experiments we observe no change in Scn11a expression in the 2 lines and have been unable to amplify Scn10a, suggesting that this channel is not abundantly expressed in the heart. Importantly, heart wall measurements, examination of contractile function (Online Table II), and survival rates were no different in CNS28+/− mice than they were in H/H animals.

Molecular Basis for CNS28 Activity

Our study identified a 435-bp segment of DNA whose deletion increases channel expression, and reporter experiments using the more physiologically relevant SCN5A promoter are consistent with this result. Our analysis of deletion constructs demonstrates that a repressor element lies within a 226-bp fragment at the 3′ end of CNS28. The 5 binding sites conserved between mouse and human in this region identified by rVista include E47, E12, E2A, MYOD, and T3R. None of these factors are good candidates to act as a repressor. However, in the 226-bp segment, we also identified a single E-box motif in the mouse and 2 in the human. Mice overexpressing Snail, which binds E-box sites in the SCN5A core promoter, exhibit a reduction in Scn5a transcript and protein.7 We observed an increase in luciferase expression following mutation of the E-box, arguing that this motif is critical for repression of SCN5A. E-boxes are typically bound by basic helix–loop–helix factors, such as HAND proteins.23 However, given the previous studies linking Snail to SCN5A, our work raises the possibility that the CNS28 E-box is a Snail-binding region and that this interaction is needed in vivo to regulate SCN5A transcript levels.

Limitations

One limitation of our study is that in order to identify elements relevant to SCN5A transcription, we choose to examine highly conserved elements between the mouse and human and replace the mouse Scn5a allele with a human SCN5A. However, in the animals we generated, potential mouse regulatory sequences, including the core promoter, are intact, and therefore the effects we see following deletion of CNS28 are in the murine context and may or may not apply to humans. However, E-boxes are evolutionarily conserved in both mouse and human promoter regions and so likely have similar functions in both species.

Summary

We hypothesize that increased sodium channel expression should translate to protection against arrhythmias that are mediated by decreased sodium channel function; settings in which this mechanism is thought to be operative include acute ischemia, especially with sodium channel block4 and the Brugada Syndrome.3,25,35 As a first test of this hypothesis, we examined the extent to which challenge with a sodium channel blocker slowed conduction in H/H and CNS28+/− mice. In this experiment, both lidocaine and flecainide prolonged PR and QRS to a similar extent; thus, with drug challenge, absolute conduction times remained shorter in the CNS28+/− animals. The further elucidation of mechanisms affecting SCN5A transcription may point to entirely novel ways in which to intervene to stabilize cardiac electrophysiological activity.

Acknowledgments

We would like to acknowledge Wei Zhang for animal care and technical assistance.

Sources of Funding

This work was supported by grants from the United States Public Health Service (HL09989, HL65962).

Disclosures

The authors have no conflicts of interest to declare.

References

18. Seibler J, Schubeler D, Fiering S, Groudine M, Bode J. DNA cassette exchange in ES cells mediated by FLP recombinase: an efficient strategy...


**Novelty and Significance**

**What Is Known?**

- Decreased cardiac sodium current, through mutations or drug block, increases susceptibility to brady- and tachyarrhythmias.
- Although in vitro experiments have identified putative regulatory elements in the cardiac sodium channel genes SCN5A, the extent to which these after channel function in vivo is unknown.

**What New Information Does This Article Contribute?**

- We identify multiple noncoding regions in SCN5A displaying cross-species sequence conservation.
- Informatic and promoter–reporter studies implicate one of these, conserved noncoding sequence 28 (CNS28), as a potential regulator of channel expression.
- We have compared SCN5A expression and function in mice with humanized cardiac sodium channels to mice that are identical, except that they lack a 435-bp fragment, which includes CNS28, in inborn 1.
- Removal of these 435 bp increases SCN5A expression and sodium current amplitude, and speeds cardiac conduction.
- Deletion of a putative Snail binding region within the 435-bp element partially relieves its repressive activity.

- Multiple lines of evidence suggest that reduced expression of the human cardiac sodium channel gene *SCN5A* can lead to arrhythmias. However, no study to date has explored the in vivo consequences of deleting potential regulatory noncoding regions of a cardiac ion gene. In this study we first identified conserved noncoding sequences (CNS) when the mouse and human genes were compared. Initial experiments identified one, CNS28, as a potential regulator of channel expression. We have previously generated mice in which the murine ortholog *Scn5a* is ablated and the human *SCN5A*-cDNA expressed in its place. In the present experiments, we generated mice in which a 435-bp region encompassing CNS28 was removed. Removing CNS28 increased *SCN5A*-mRNA, protein, and sodium current, and was associated with enhanced conduction (decreased PR and QRS intervals). Promoter–reporter experiments showed that removal of an E-box in CNS28 increased reporter activity. These findings support the hypothesis that variable expression of cardiac ion channels can alter the electrophysiological properties of the intact heart. Understanding the mechanisms underlying ion channel regulation could lead to potential new therapies for arrhythmias.
Informatic and Functional Approaches to Identifying a Regulatory Region for the Cardiac Sodium Channel

Thomas C. Atack, Dina Myers Stroud, Hiroshi Watanabe, Tao Yang, Lynn Hall, Susan B. Hipkens, John S. Lowe, Brenda Leake, Mark A. Magnuson, Ping Yang and Dan M. Roden

Circ. Res. 2011;109:38–46; originally published online May 12, 2011; doi: 10.1161/CIRCRESAHA.110.235630

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/109/1/38

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2011/05/12/CIRCRESAHA.110.235630.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
**Supplement Material**

**Expanded Methods**

**Identification of potential regulatory regions**

To identify CNS elements, we compared the human SCN5A locus with its mouse ortholog using the VISTA Genome Browser (http://pipeline.lbl.gov/cgi-bin/gateway2).1,2 Each of 92 CNS elements identified was then PCR amplified and assayed for activity as described below. Those showing >5-fold increase in reporter activity in luciferase assays were then analyzed for potential muscle-specific transcriptional regulatory modules using the M-SCAN algorithm (http://www.cisreg.ca/cgi-bin/mscan/MSCAN).3,4 For identification of potential repressive transcription factors in CNS28 we used rVista (http://rvista.dcode.org/) to compare human and mouse sequences for conserved transcription factor binding sites.5,6

**Generation of reporter constructs**

Three sets of reporter constructs were made. Initial screening experiments examined luciferase reporter activity of CNS constructs in the pGL3-Promoter vector (Promega). Subsequently, we conducted a series of experiments examining the effect of CNS28 on the activity of the SCN5A core promoter and CNS28 truncation constructs with the previously identified alternate promoter.7 Each of the 92 CNS identified in the initial VISTA scan was PCR-amplified thus generating a series of luciferase fusion constructs (pGL3-CNS1 through pGL3-CNS92). The PCR reactions used forward primers containing an *NheI* recognition sequence at the 5' end and reverse primers containing an *XhoI* recognition sequence (See Supplemental Table 1). PCR products were digested with *NheI* and *XhoI*, and the fragments were subcloned into the pGL3-Promoter vector.

The starting point for generating CNS28/SCN5A promoter constructs, was the pGL3-Basic vector containing the full length SCN5A promoter we previously generated.8 CNS28 was amplified from human genomic DNA obtained from Vanderbilt University’s Polymorphism Discovery Core, using modified versions of the CNS28 primers, F_ and R_ (Table 1), and the Expand High Fidelity Kit (Roche) following the protocol provided. CNS28 was inserted into the vector using the *SalI*/BamHI sites downstream of the luciferase cDNA. All constructs were verified by direct sequencing.

Experiments were also conducted to analyze CNS28 truncation constructs. Human and mouse CNS28 sequences were compared and scanned for conserved transcription factors using rVista 2.0.5,6 Five fragments were amplified based on the location of regulatory elements from the rVista analysis. DC1-F, DC2-F, and DC3-F (Table 1) remove the first 50, 150, and 280 base pairs from CNS28 respectively. The universal reverse primer, DC-R, as well as DC0-F and DC4-F (Table 1) amplify the alternate promoter previously identified,7 with and without the whole CNS28 fragment respectively. PCR fragments were amplified using the Expand Long kit (Roche) with CNS28- mouse genomic DNA as the template. The cycling conditions used were an initial denaturing step of 94°C for 2' followed by 35 cycles of 94°C for 20", 65°C for 30", and 72°C for 2' and a final 7' extension at 72°C. PCR fragments were digested with *KpnI/*BglII and inserted into the pGL3-Basic vector (Promega). All constructs were verified by direct sequencing.

**Mutagenesis**

Mutagenesis on DC3 was performed using the QuickChange II XL kit (Stratagene) using the primers listed in Table 1. The standard protocol provided with the kit was followed and the construct was verified by direct sequencing.

**Reporter assays**
Experiments were conducted as previously described in CHO cells\textsuperscript{8,9} and in cardiomyocytes\textsuperscript{8-10} isolated from 1-2 day old mice. The pGL3-CNS/luciferase fusion genes (125 ng DNA) were transfected into the neonatal mouse cardiac cells using FuGENE 6 (Roche), and into CHO cells using lipofectamine reagent (Invitrogen). The pGL3-Promoter plasmid was tested in each experiment and its activity level served as the no insertion control and its activity was designated as 100%. The SCN5A Promoter and SCN5A Promoter-CNS28/luciferase genes (500 ng DNA) were transfected into both cell lines using FuGENE 6 (Roche) with the construct containing CNS28 designated as 100% activity. The CNS28 truncation constructs containing the alternate mouse promoter (250 ng DNA) were transfected into both cell lines using FuGENE 6 (Roche). The construct containing the full CNS28 sequence was designated 100% activity. In each experiment, the pRL-TK plasmid (5-10 ng), encoding Renilla luciferase, was co-transfected to normalize for experimental variability caused by differences in cell viability or transfection efficiency. Luminescence was measured after 48 hr transfection by using Dual-Luciferase Reporter Assay System (Promega). The DC3 mutant transfection and luciferase assay were performed in an identical manner to the truncation constructs.

**Generation of CNS28-KO mice**

Mouse embryonic stem (ES) cells in which a region of the Scn5a gene was modified to allow RMCE to be used to easily generate mice containing allelic variant of human sodium channels under control of the Scn5a regulatory sequences.\textsuperscript{11} Using this approach, we generated H/H mice in which the targeted region was replaced by the human SCN5A full-length cDNA. For the present experiments, we modified the original H exchange construct to delete bases 1720 to 2154 (that encompass the CNS28 region), and then performed RMCE as previously described to generate CNS28\textsuperscript{+/-} animals. After removal of a hygromycin resistance cassette by breeding to a FlpE-expressing line, mice were then interbred to generate CNS28\textsuperscript{-/-} animals.\textsuperscript{11} Experiments began after three backcrosses using littermate H/H mice as controls. All experiments performed on mice were approved by the institutional animal care and use committee.

**Quantitative Real Time RT-PCR**

Total mRNA from adult mice atria or ventricles was isolated using the TRIzol method (Invitrogen) and cDNA was prepared using the Transcriptor First Strand cDNA Synthesis kit (Roche). Quantitative Real Time RT-PCR (qPCR) on SCN5A was performed with TaqMan probes targeting human SCN5A (Hs00165693_m1) using beta actin (Mm00607939_s1) as a reference gene. qPCR targeting mouse Scn1b was performed using TaqMan probe (Mm00441210_m1) with Hprt1 (Mm00446968_m1) as the reference gene. The reactions were performed using the TaqMan Fast Universal PCR Master Mix in a 7900HT thermocycler (Applied Biosystems). Cycling conditions were an initial step of 15” at 95° C, followed by 40 cycles of 1” at 95° C and 10” at 60° C. Standard dilutions of plasmids containing the target genes were used to quantitate the amounts of mRNA present in our samples. Relative mRNA amounts were calculated comparing human SCN5A to mouse beta actin levels and were normalized to the levels in H/H mice. Analysis was performed using SDS 2.2.2 software (Applied Biosystems)

**Western blotting**

Western blot analysis of Na\textsubscript{v}1.5 (SCN5A protein) and calnexin was performed using 100ug of whole heart protein extract. Blots were cut to separate the SCN5A and calnexin bands and were stained with anti-SCN5A antibody (Alomone ASC-005 1:250 dilution) or anti-calnexin antibody (Stressgen SPA860 1:2000 dilution) respectively. The secondary antibody was anti-rabbit IgG HRP-conjugate (Promega W401B 1:10,000 dilution). Films were scanned on a standard desktop scanner and band density was measured using ImageJ, freely available from the NIH. Relative protein levels were
calculated for each sample by comparing band densities for SCN5A and calnexin
signals.

**Electrocardiogram (ECG) recordings and lidocaine challenge**

ECGs and drug challenges were recorded as previously described. In brief, adult mice were anesthetized with isoflurane vapor adjusted to maintain the light anesthesia and a constant heart rate throughout the recording procedure. Electrodes were inserted subcutaneously in each limb and baseline recordings were obtained over 5 minutes. For lidocaine challenge, ECGs were recorded at baseline as described above and for an additional 20 minutes after intraperitoneal injection of lidocaine 40 mg/kg (Sigma). ECG signals were averaged over 10 second epochs and intervals were obtained from the signal averaged tracings using a custom signal averaging program using National Instruments LabView.

**Isolation of mouse ventricular cardiomyocytes and sodium current recordings**

Adult H/H and CNS28-/- mouse cardiac ventricular myocytes were isolated by a modified collagenase/protease method. After intraperitoneal injection of 500 IU of heparin, adult mice were anesthetized using inhaled isoflurane/oxygen mixture, hearts excised, and their aortae rapidly cannulated and perfused with modified Tyrode's solution (MTS) for 3 min followed by MTS containing collagenase (Liberase Blendzyme-4, Roche, 0.04 mg/ml) for 5–7 min at a constant pressure of 80 mmHg and temperature of 34 ºC. The MTS contained (in mmol/l) NaCl 130, HEPES 10, glucose 10, KCl 5.4, MgCl2 1.2, NaH2PO4, 2,3-butanedione monoxime 10, pH of 7.2. The digested ventricles were minced in MTS containing 1 mg/ml bovine serum albumin and 0.2 mmol/l CaCl2 and triturated by gently pipetting. The resulting solution was strained and the myocytes allowed to sediment in MTS of increasingly higher Ca2+ concentrations (0.2, 0.5, and 1 mmol/l). This procedure routinely yielded 60–80% rod-shaped, Ca2+-tolerant myocytes that were used for the electrophysiology studies.

To control INa, external sodium concentration was lowered to 5 mM, wide-tip electrodes with tip resistance <1 MΩ were used, and experiments were conducted at 18ºC. INa was recorded using whole-cell voltage clamp. The pipette-filling (intracellular) solution contained (in mmol/L): NaF 10, CsF 110, CsCl 20, EGTA 10, HEPES 10, with a pH of 7.4 adjusted with CsOH. The bath (extracellular) solution had (in mmol/L): NaCl 5, CsCl 5, TEA-Cl 135, CaCl2 0.1, MgCl2 1, HEPES 10, and glucose 10, with a pH of 7.4 adjusted by CsOH. To eliminate L- and T-type inward calcium currents, 0.5 µM nisoldipine and 200 µM NiCl2 were added to the bath solution. Data acquisition was carried out using an Axopatch 200B patch-clamp amplifier and pCLAMP version 9.2 software (MDS Inc., Mississauga, Ontario, Canada). Currents were filtered at 5 kHz (-3 dB, four-pole Bessel filter) and digitized using an analog-to-digital interface (Digidata 1322A, MDS Inc.). To minimize capacitive transients, capacitance, and series resistance were adjusted to 70–85%. The holding potential was -120 mV for all experiments, and details of the pulse protocols are presented schematically in the figures. INa densities in H/H and CNS28-/- mice were compared as pA/pF after normalization to cell sizes which were generated from the cell capacitance calculated by Membrane Test (OUT 0) in pClamp 9.2. Electrophysiologic data were analyzed using Clampfit version9.2 (Axon Instruments), and figures were prepared by using Origin 7.0 (OriginLab Corp., Northampton, MA, USA). Current-voltage relations for steady-state activation and inactivation were fit with the Boltzmann equation I/Imax = (1 + exp[(V - V1/2)/k])-1 to determine the membrane potential for half-maximal activation (V1/2-activation) and inactivation (V1/2-inactivation).

**Echocardiogram**

Transthoracic echocardiograms were performed on resting conscious mice at the Murine Cardiovascular Core, Vanderbilt University as previously described. Signals
were acquired using a 15-MHz transducer (Sonos 5500 system, Agilent) and analyzed by a sonographer who was blind to the genotype.

Data analysis

Results are presented as mean±SE, and statistical comparisons were made using the unpaired Student’s t test. A value of P<0.05 was considered statistically significant.
Online Figure I
Selection of deletion construct primer locations. Using the rVista output of the human and mouse CNS28 alignments as a guide, locations for the forward primer of the deletion constructs were chosen within the gaps between clusters of predicted transcription factor binding sites.
Online Figure II

Relative amount of *Scn1b*. Using Real-Time PCR we analyzed amounts of *Scn1b* transcript relative to *Hprt1* levels. There was no significant difference between H/H mice and CNS28<sup>−/−</sup> mice.
<table>
<thead>
<tr>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNS-2F: GCGTGCTAGCCGAGTGTTGATGTCTCCGCCCT</td>
<td>CNS-2R: AGATCTCGAGTACTGGAAGTCAATGCTTCGGG</td>
<td>5'-end</td>
</tr>
<tr>
<td>CNS-3F: GCGTGCTAGCCTGGCATGGCAAAATGGGTACAT</td>
<td>CNS-3R: AGATCTCGAGCAACTGTTTTCCCATC TCTACTGC</td>
<td>5'-end</td>
</tr>
<tr>
<td>CNS-6F: GCGTGCTAGCTCTCCAGGAGACATTACACAGAC</td>
<td>CNS-6R: AGATCTCGAGCGTTCTTTCATTTTAATAAGG</td>
<td>5'-end</td>
</tr>
<tr>
<td>CNS-7F: GCGTGCTAGCCAGCTCTACCTGCAATGGTATT</td>
<td>CNS-7R: AGATCTCGAGATAGATGTGTGGTGGTATTTCA</td>
<td>5'-end</td>
</tr>
<tr>
<td>CNS-8F: GCGTGCTAGCGGGTGGGGTTGAAACGTTGACACT</td>
<td>CNS-8R: AGATCTCGAGGATTGGCTAATTGTTTCTTAGG</td>
<td>5'-end</td>
</tr>
<tr>
<td>CNS-9F: GCGTGCTAGCCCTCCAGGAGACATTACACAGAC</td>
<td>CNS-9R: AGATCTCGAGAACTGGTATTTAAAGGGTCAGC</td>
<td>5'-end</td>
</tr>
<tr>
<td>CNS-10F: GCGTGCTAGCCGCTGGAGGCGGAGGATTAAGGT</td>
<td>CNS-10R: AGATCTCGAGGCTGCTTGGCAGTGGATTTCA</td>
<td>5'-end</td>
</tr>
<tr>
<td>CNS-11F: GCGTGCTAGCTCTCCAGGAGCGGCAGACTCT</td>
<td>CNS-11R: AGATCTCGAGCAGCTGATCCCTGTCCGCTTCC</td>
<td>intron1</td>
</tr>
<tr>
<td>CNS-12F: GCGTGCTAGCCCTCCAGGAGCGGCAGACTCT</td>
<td>CNS-12R: AGATCTCGAGCCAGCTGATCCCTGTCCGCTTCC</td>
<td>intron1</td>
</tr>
<tr>
<td>CNS-13F: GCGTGCTAGCCCTCCAGGAGCGGCAGACTCT</td>
<td>CNS-13R: AGATCTCGAGCCAGCTGATCCCTGTCCGCTTCC</td>
<td>intron1</td>
</tr>
<tr>
<td>CNS-14F: GCGTGCTAGCCCTCCAGGAGCGGCAGACTCT</td>
<td>CNS-14R: AGATCTCGAGCCAGCTGATCCCTGTCCGCTTCC</td>
<td>intron1</td>
</tr>
<tr>
<td>CNS-15F: GCGTGCTAGCCCTCCAGGAGCGGCAGACTCT</td>
<td>CNS-15R: AGATCTCGAGCCAGCTGATCCCTGTCCGCTTCC</td>
<td>intron1</td>
</tr>
<tr>
<td>CNS-16F: GCGTGCTAGCCCTCCAGGAGCGGCAGACTCT</td>
<td>CNS-16R: AGATCTCGAGCCAGCTGATCCCTGTCCGCTTCC</td>
<td>intron1</td>
</tr>
<tr>
<td>CNS-17F: GCGTGCTAGCCCTCCAGGAGCGGCAGACTCT</td>
<td>CNS-17R: AGATCTCGAGCCAGCTGATCCCTGTCCGCTTCC</td>
<td>intron1</td>
</tr>
<tr>
<td>CNS-18F: GCGTGCTAGCCCTCCAGGAGCGGCAGACTCT</td>
<td>CNS-18R: AGATCTCGAGCCAGCTGATCCCTGTCCGCTTCC</td>
<td>intron1</td>
</tr>
<tr>
<td>CNS-19F: GCGTGCTAGCCCTCCAGGAGCGGCAGACTCT</td>
<td>CNS-19R: AGATCTCGAGCCAGCTGATCCCTGTCCGCTTCC</td>
<td>intron1</td>
</tr>
<tr>
<td>CNS-20F: GCGTGCTAGCCCTCCAGGAGCGGCAGACTCT</td>
<td>CNS-20R: AGATCTCGAGCCAGCTGATCCCTGTCCGCTTCC</td>
<td>intron1</td>
</tr>
</tbody>
</table>
CNS-65F GCGTGCTAGCCTCATCAGGTAAAATCAGGTTAAAACAC | CNS-65R AGATCTCGAGGAAAAGCAGTCCACTATCTTTGG | intron6
CNS-63F GCGTGCTAGCCCTGAGTGGTTTCGGGTAGGGT | CNS-63R AGATCTCGAGAGATCCTGCTGCCAGTGTCCCCCAT | intron7
CNS-62F GCGTGCTAGGCCTCTAGGCTATGGCTATTTG | CNS-62R AGATCTCGAGAGATCCTGCTGCCAGTGTCCCCCAT | intron9
CNS-84F GCGTGCTAGCCCTCAGGGAGCCCTCTATACAGCAGGTTGTGT | CNS-84R AGATCTCGAGTTGTCCCCTGTCCCCTTTGGACTGAGT | intron9
CNS-61F GCGTGCTAGCCCTTACTTATTCACCTCCTCTCTCTAT | CNS-61R AGATCTCGAGACCTACCCCTAAAATGAGGA | intron12
CNS-33F GCGTGCTAGGAGCTGCCTGAGGTGAAAATCAGGTTAAAACAC | CNS-33R AGATCTCGAGGACCCCAATCTTGTTTGGTCCTT | intron14
CNS-58F GCGTGCTAGCCTACAGGGAGCCCTCTATACAGCAGGTTGTGT | CNS-58R AGATCTCGAGGAAAAGCAGTCCACTATCTTTGG | intron14
CNS-59F GCGTGCTAGGCCTCTAGGCTATGGCTATTTG | CNS-59R AGATCTCGAGGACCCCAATCTTGTTTGGTCCTT | intron14
CNS-60F GCGTGCTAGCCCTCAGGGAGCCCTCTATACAGCAGGTTGTGT | CNS-60R AGATCTCGAGGAAAAGCAGTCCACTATCTTTGG | intron14
CNS-83F GCGTGCTAGCCCTGAGTGGTTTCGGGTAGGGT | CNS-83R AGATCTCGAGAGATCCTGCTGCCAGTGTCCCCCAT | intron14
CNS-57F GCGTGCTAGGCCTCTAGGCTATGGCTATTTG | CNS-57R AGATCTCGAGGACCCCAATCTTGTTTGGTCCTT | intron14
CNS-34F GCGTGCTAGGAGCTGCCTGAGGTGAAAATCAGGTTAAAACAC | CNS-34R AGATCTCGAGGAAAAGCAGTCCACTATCTTTGG | intron14
CNS-56F GCGTGCTAGGCCTCTAGGCTATGGCTATTTG | CNS-56R AGATCTCGAGGACCCCAATCTTGTTTGGTCCTT | intron18
CNS-35F GCGTGCTAGGCCTCTAGGCTATGGCTATTTG | CNS-35R AGATCTCGAGGACCCCAATCTTGTTTGGTCCTT | intron20
CNS-36F GCGTGCTAGGCCTCTAGGCTATGGCTATTTG | CNS-36R AGATCTCGAGGACCCCAATCTTGTTTGGTCCTT | intron20
CNS-51F GCGTGCTAGGCCTCTAGGCTATGGCTATTTG | CNS-51R AGATCTCGAGGACCCCAATCTTGTTTGGTCCTT | intron20
CNS-52F GCGTGCTAGGCCTCTAGGCTATGGCTATTTG | CNS-52R AGATCTCGAGGACCCCAATCTTGTTTGGTCCTT | intron20
CNS-53F GCGTGCTAGGCCTCTAGGCTATGGCTATTTG | CNS-53R AGATCTCGAGGACCCCAATCTTGTTTGGTCCTT | intron20
CNS-54F GCGTGCTAGGCCTCTAGGCTATGGCTATTTG | CNS-54R AGATCTCGAGGACCCCAATCTTGTTTGGTCCTT | intron20
CNS-55F GCGTGCTAGGCCTCTAGGCTATGGCTATTTG | CNS-55R AGATCTCGAGGACCCCAATCTTGTTTGGTCCTT | intron20
CNS-37F GCGTGCTAGGCCTCTAGGCTATGGCTATTTG | CNS-37R AGATCTCGAGGACCCCAATCTTGTTTGGTCCTT | intron22
CNS-50F GCGTGCTAGGCCTCTAGGCTATGGCTATTTG | CNS-50R AGATCTCGAGGACCCCAATCTTGTTTGGTCCTT | intron26
CNS-38F GCGTGCTAGGCCTCTAGGCTATGGCTATTTG | CNS-38R AGATCTCGAGGACCCCAATCTTGTTTGGTCCTT | 3'-end
CNS-39F GCGTGCTAGGCCTCTAGGCTATGGCTATTTG | CNS-39R AGATCTCGAGGACCCCAATCTTGTTTGGTCCTT | 3'-end
CNS-40F GCGTGCTAGGCCTCTAGGCTATGGCTATTTG | CNS-40R AGATCTCGAGGACCCCAATCTTGTTTGGTCCTT | 3'-end
CNS-41F GCGTGCTAGGCCTCTAGGCTATGGCTATTTG | CNS-41R AGATCTCGAGGACCCCAATCTTGTTTGGTCCTT | 3'-end
| CNS-42F GCGTGCTAGCCCCTCACCCCTTATCCAAATG | CNS-42R AGATCTCGAGAACTTACTAGTTGGCCCATTCATCA | 3'-end |
| CNS-43F GCGTGCTAGCCCTGAGGAACAGACCTCC | CNS-43R AGATCTCGAGTGACCTCATTTCCCTTTTCTTG | 3'-end |
| CNS-44F GCGTGCTAGCCCTCCAAGAGCCTTCACTTCCACT | CNS-44R AGATCTCGAGAGGTGGTGGCTGGCCTGGAAGG | 3'-end |
| CNS-45F GCGTGCTAGCCCTCTCTCATCCCTCCCTGACTCT | CNS-45R AGATCTCGAGTGTCCACTCCCCGAGTCACAC | 3'-end |
| CNS-46F GCGTGCTAGCCCTCTTTTAAAGAGAAGCGCGG | CNS-46R AGATCTCGAGTGACCTCGAGACAGAGACCGAAGGGGAG | 3'-end |
| CNS-47F GCGTGCTAGCCCTAGCGGAAGCGCCATCTCATGTGGTA | CNS-47R AGATCTCGAGACTCCCTGATCCATCTGC | 3'-end |
| CNS-48F GCGTGCTAGCCCTAGCGGAAGCGCCATCTCATGTGGTA | CNS-48R AGATCTCGAGACCACACCACCTTGCCTCCTCTCT | 3'-end |
| CNS-49F GCGTGCTAGCCCTTGGTGGGAGGCGACGATATC | CNS-49R AGATCTCGAGTGTTCTGGCAAGGCTGGGAG | 3'-end |
| CNS-80F GCGTGCTAGCCCTACCTAGGACTTATCCACAGA | CNS-80R AGATCTCGAGATCCATTCTTCTTCTTGCACTAC | 3'-end |
| CNS-81F GCGTGCTAGCCCTGAGCGCCTGCTTCTTCTTTT | CNS-81R AGATCTCGAGCCCTTCTTTCCGAGTGTTATTA | 3'-end |
| CNS-92F GCGTGCTAGCCACAGGGAGATGCCTCAAATCC | CNS-92R AGATCTCGAGGGGAATGCCAGGCTTAAAGTA | 3'-end |
## Online Table II. Echocardiogram Measurements of Adult Mice

<table>
<thead>
<tr>
<th></th>
<th>H/H (n=9)</th>
<th>CNS28&lt;sup&gt;-/-&lt;/sup&gt; (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Septal Wall (mm)</td>
<td>0.72 ± 0.02</td>
<td>0.74 ± 0.05</td>
</tr>
<tr>
<td>Posterior Wall (mm)</td>
<td>2.98 ± 0.11</td>
<td>3.06 ± 0.19</td>
</tr>
<tr>
<td>Left Ventricle Systole (mm)</td>
<td>0.58 ± 0.02</td>
<td>0.52 ± 0.03</td>
</tr>
<tr>
<td>Left Ventricle Diastole (mm)</td>
<td>1.50 ± 0.06</td>
<td>1.65 ± 0.11</td>
</tr>
<tr>
<td>Fractional Shortening %</td>
<td>49.7 ± 0.9</td>
<td>46.0 ± 1.9</td>
</tr>
</tbody>
</table>
References


