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Voltage-gated sodium channels are required for heart development in zebrafish

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Abstract

Rationale—Voltage-gated sodium channels initiate action potentials in excitable tissues. Mice in which *Scn5A* (the predominant sodium channel gene in heart) has been knocked out die early in development with cardiac malformations by mechanisms which have yet to be determined.

Objective—Here we addressed this question by investigating the role of cardiac sodium channels in zebrafish heart development.

Methods and Results—Transcripts of the functionally-conserved *Scn5a* homologs *scn5Laa* and *scn5Lab* were detected in the gastrulating zebrafish embryo and subsequently in the embryonic myocardium. Antisense knockdown of either channel resulted in marked cardiac chamber dysmorphogenesis and perturbed looping. These abnormalities were associated with decreased expression of the myocardial precursor genes *nkx2.5*, *gata4*, and *hand2* in anterior lateral mesoderm and significant deficits in the production of cardiomyocyte progenitors. These early defects did not appear to result from altered membrane electrophysiology, as prolonged pharmacological blockade of sodium current failed to phenocopy channel knockdown. Moreover, embryos grown in calcium channel blocker-containing medium had hearts that did not beat but developed normally.

Conclusions—These findings identify a novel, and possibly non-electrogenic, role for cardiac sodium channels in heart development.

Keywords

Ion Channels; Heart Development; Zebrafish; *scn5La*

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Disclosures
None

Introduction

The Na_v1 family of voltage-gated sodium channels are multi-protein complexes that account for the initial upstroke (phase 0) of the action potential in neurons, myocytes, and other excitable cells by permitting a rapid influx of Na^+ ions.[1] Ten distinct pore-forming (α) subunit genes (*SCNxA*) have been cloned,[1–3] and *SCN5A* encodes $Na_v1.5$, the predominant sodium channel isoform in myocardium.[4]

Perturbed expression or function of $Na_v1.5$ in patients can cause a range of phenotypes including the long QT syndrome, Brugada syndrome, progressive cardiac conduction system disease, and atrial arrhythmias.[5, 6] Mice heterozygous for *Scn5a* deletion display slow conduction and susceptibility to ventricular tachycardia.[7] By contrast, $Scn5a^{-/-}$ homozygotes die between embryonic day 10 (E10) and E11 with abnormalities of ventricular morphogenesis, indicating that *Scn5a* is also required for normal development.[7] The mechanisms underlying these defects have not been determined.

Here we used zebrafish to examine the role of sodium channels in the developing heart. Zebrafish embryos are optically transparent and externally fertilized, facilitating the study of early organ formation. Genetic manipulation is readily achieved using antisense morpholinos, and embryos are also permeable to small molecule drugs placed in their medium. The stages of zebrafish cardiac development have been well-delineated: cardiac precursors are located at the blastula margin at 5 hours post fertilization (hpf).[8] These bilateral precursors undergo a complex series of movements that result in the formation of a cardiac cone and subsequently a beating heart tube by 22–24hpf.[9, 10] The tube then loops to form a two-chambered heart and the ventricular wall begins to thicken concentrically between 48–72hpf.[9, 10] Our findings identify a previously-unappreciated role for voltage-gated sodium channels in early cardiac development.

Materials and methods

The following zebrafish strains were used in this study: AB, TuAB, *Tg(cmlc2:GFP)*, *Tg (cmlc2:DsRed2-nuc)*. Whole-mount *in situ* hybridization was performed using digoxigenin-labeled probes. Gene knockdown was achieved by morpholino antisense oligonucleotides (Gene Tools) designed to inhibit translation of mRNAs or disrupt splicing of pre-mRNAs. Drugs and toxins were delivered to embryos by bath exposure or by pressure injection directly into the yoke sac, trunk circulation, sinus venosus, or pericardial sac, depending on the experiment and developmental stage. Transcript levels were quantified using Power SYBR Green (Applied Biosystems). Confocal images of live embryos were captured using a Zeiss LSM 510 Confocal Microscope System equipped with either 20x or 40x objective lenses. Image analysis and cell counts were performed using Image J software. Photographs of embryos were adjusted for brightness and contrast in Adobe Photoshop and/or Microsoft Powerpoint and identical adjustments were made for active treatment groups and controls. Statistical analyses were performed as indicated in the text and figure legends.

An expanded Materials and methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Results

Cardiac sodium channels in zebrafish

Two *SCN5A* orthologs have been cloned in zebrafish[11] (see also Online Figure I) and are termed *scn5Laa* and *scn5Lab*. These encode typical voltage-gated sodium channels sharing 60–65% amino acid identity with SCN5A (Online Figure II, Online Figure III), with even greater

conservation in important functional domains including the transmembrane segments, voltage-sensors, pore loops, inactivation gate, and C-terminus (Online Figure III). Expression of full-length *scn5Laa* and *scn5Lab* in CHO cells produced typical voltage-gated sodium currents (Figure 1A, B). Transcripts of both genes were detected in gastrulating embryos prior to the expression of early cardiogenic transcription factors and well in advance of myocardial differentiation (Figure 1C). Both sodium channel genes were expressed in the heart, brain, and spinal cord at 52 −104hpf by *in situ* hybridization (Figure 1D, E).

Knockdown of zebrafish cardiac sodium channels results in defects of cardiac morphogenesis

To investigate the role of *scn5Laa* and *scn5Lab* in development, we used multiple translation- and splice-blocking morpholino-antisense oligonucleotides (morpholinos) that specifically target each gene as well as 5-nucleotide mismatch control morpholinos (Online Figure IV–Online Figure V, Online Table I). Knockdown of either *scn5Laa* or *scn5Lab* in *Tg (cmlc2:GFP)* zebrafish, a transgenic line expressing green fluorescent protein in the myocardium,[12] resulted in embryos with marked defects in cardiac development and function compared to embryos injected with equivalent doses of control morpholinos (Figure 2, Online Table II). Dual knockdown of *scn5Laa* and *scn5Lab* produced embryos with cardiac defects that appeared more severe than those resulting from knockdown of either gene alone, primarily with respect to ventricular development (Figure 2B). As an additional control, we knocked-down the cardiac potassium channel *zerg* which caused arrhythmias by 48hpf but did not perturb development (Figure 2, Online Table II).

Non-specific morpholino toxicity or cell death can be minimized by concomitant knockdown of the zebrafish *p53* gene.[13] Co-injection of sodium channel and *p53* morpholinos resulted in embryos with increased head size and overall body length but equally severe defects in cardiac development compared to embryos injected with sodium channel morpholinos alone (Figure 2, Online Figure VI). Acridine orange staining of morphant embryos identified no apoptotic cells in the dysmorphic, hypoplastic sodium channel morphant hearts (Online Figure VII).

Sodium channels are required for the production of physiological numbers of embryonic cardiomyocytes

Scn5Laa morphant hearts displayed marked abnormalities of both atrial and ventricular chamber morphogenesis and looping at 58hpf (Figure 3A, B). At 104hpf, when growth of the zebrafish heart is achieved primarily by cardiomyocyte proliferation, *scn5Laa* morphant cardiac chambers remained small (Figure 3C, D). While control embryo hearts added cardiomyocytes to the interior of the ventricular wall to form trabeculae, *scn5Laa* morphant ventricles remained a single cell layer lacking trabeculated myocardium (Figure 3E–H). These findings indicate that sodium channel knockdown compromises both early chamber formation and normal patterned growth of the ventricle. Quantification of cardiomyocyte number in *Tg (cmlc2:DsRed2-nuc)* zebrafish revealed significant deficits in *scn5Laa* and *scn5Lab* morphants at 60–62hpf (Figure 3I). Dual *scn5Laa*/*scn5Lab* morphants had the fewest number of cardiomyocytes (40% less than controls) (Figure 3I).

Knockdown of sodium channels disrupts the expression of cardiogenic transcription factors in anterior lateral mesoderm

The expression of *scn5Laa* and *scn5Lab* during gastrulation and the diminished production of cardiomyocytes in morphant embryos together suggested that sodium channels may be required for the specification of normal numbers of cardiac progenitor cells. To test this hypothesis, we used *in situ* hybridization to examine gene expression in the heart-forming region of anterior lateral plate mesoderm (ALPM), a population of undifferentiated cells with cardiac potential demarcated by the expression of *nkx2.5* and the overlapping expression domains of *gata4* and

hand2.[14] Compared to control embryos, *scn5Laa* morphants displayed decreased expression of all three transcription factors in ALPM at the 6-somite stage (Figure 4A, C). Somite staging was used to confirm that the observed differences were not the result of developmental delay. Although *gata5* is a potent inducer of *nkx2.5* expression in zebrafish,[15] *gata5* expression levels were indistinguishable in *scn5Laa* morphant and control embryos (Figure 4A). These findings were independently corroborated by real-time RT-PCR (Figure 4B). Similar to *scn5Laa*, knockdown of *scn5Lab* also resulted in mispatterning of anterior lateral mesoderm with decreased expression of both *nkx2.5* and *gata4* (Figure 4D). The effect of *scn5Laa* and *scn5Lab* morpholinos on *nkx2.5* expression at 6–8 somites was dose-dependent (Figure 4E). These results indicate that zebrafish cardiac sodium channels are required for the normal expression of cardiac fate-determining genes in anterior lateral mesoderm and for specification of appropriate numbers cardiac progenitor cells during development.

Sodium channel knockdown results in reduced numbers of differentiating cardiomyocytes

At the 16-somite stage, *scn5Laa* and *scn5Lab* morphants displayed reduced expression not only of *nkx2.5* but of the sarcomeric genes *cmlc2* and *vmhc* (Figure 5A). Moreover, examination of the number of somites (marked by *vmhc* expression) revealed that reductions in the size of the field of differentiating cardiomyocytes were not due to a global developmental delay. To assess the magnitude of the reduction in differentiating cardiomyocytes, we quantified total cardiac cell number in the cardiac cone of *Tg(cmlc2:GFP)* zebrafish at the 22-somite stage, the earliest timepoint when we could detect fluorescence in this line of transgenic zebrafish. At this stage, we found a significant reduction in the total number of newly differentiated myocytes in *scn5Laa* morphants compared to control embryos (Figure 5C).

Voltage-gated sodium channels may regulate early cardiac development independent of membrane electrophysiology

Prior studies in mice and chicks suggest that coordinated electrical activity in the early heart does not require fast sodium current (I_{Na}).[16–18] Consistent with these prior observations, delivery of sodium channel blockers (e.g. TTX) and activators (e.g. ATX) directly into the pericardial space of zebrafish embryos failed to perturb heart-beating at early stages but caused conduction abnormalities at later stages (Online Figure VIII, Online Table III, Online Movie I–Online Movie IV). By contrast, the L-type calcium channel blocker nisoldipine silenced the heart at every stage examined. Therefore, we reared embryos in a high concentration of nisoldipine (10μM) from cleavage-stages through 78hpf. Nisoldipine-treated embryos failed to initiate heart beating at any stage examined but displayed normal cardiac morphogenesis through 60hpf and normal cardiomyocyte number compared to vehicle-treated embryos (Figures 6A, B, Table 1). This result is similar to that previously observed in zebrafish harboring mutations in cardiac troponin T (*silent heart*) in which heart formation is unaffected despite an absence of both contractility and circulation.[19] These findings demonstrate that neither coordinated electrical activity nor beating is required for the earliest steps of cardiac development.

We next hypothesized that sodium current (I_{Na}) may have an important role in early cardiac development prior to the onset of heart-beating. However, high concentrations of a range of drugs that activate or block I_{Na} had no effect on cardiac specification or differentiation when delivered to embryos in the bath solution or by microinjection (Figure 6C, Table 1). Importantly, TTX-treated embryos displayed complete neuromuscular paralysis at the end of the treatment period (~28hpf) (Table 1, Online Video V), and treatment with channel activators (e.g. ATX II, veratridine) resulted in neuromuscular hyperexcitability (Table 1, Online Video VI). These findings indicate adequate compound penetration and confirmed that these toxins interact with other voltage-gated sodium channels *in vivo*, even at early stages. Early cardiogenesis also proceeded normally in embryos reared from the 1 cell stage in sodium-free

media, another approach that inhibits inward I_{Na} (Figure 6D). Taken together, these results indicate that sodium channel expression but not sodium current is required for early cardiac development in zebrafish.

Discussion

Na_v1 voltage-gated sodium channels underlie the upstroke of the action potential and are thus the principal determinants of membrane excitability in the nervous system, skeletal muscle, heart and other tissues.[1] In the present study, we identified a previously-unappreciated role for cardiac-type sodium channels in the developing heart. We detected expression of *scn5Laa* and *scn5Lab* in the gastrulating embryo, prior to the differentiation of excitable tissues. Using both translation- and splice-blocking morpholino antisense oligonucleotides, we found that reduced expression of either isoform disrupted early cardiogenic gene expression in anterior lateral mesoderm and compromised the generation of sufficient numbers of progenitor cells for normal cardiac morphogenesis. We and others demonstrated that hearts that do not beat still display normal early development.[19] Moreover, pharmacological manipulation of sodium current (I_{Na}) *in vivo* generated neuromuscular phenotypes but did not affect cardiac gene expression or morphogenesis, suggesting that sodium channel expression but not I_{Na} is required for cardiac development. These findings indicate that voltage-gated sodium channels have important roles in vertebrate heart development and support the hypothesis that these roles are mediated by non-electrogenic functions of the channel complex.

Voltage-gated sodium channels are required for early cardiac gene expression

A key defect we observed in sodium channel morphant embryos was a deficiency in the expression of multiple transcription factors that contribute to the specification of cardiac progenitor cells from anterior lateral mesoderm. Although reduced in number, these progenitors appear to normally differentiate into nascent cardiomyocytes. Early cardiogenic mesoderm is distinguished by the overlapping expression of a group of highly conserved regulatory genes belonging to the NK2, GATA, MEF2, T-box, and basic helix loop helix (bHLH) families of transcription factors.[20, 21] The markedly reduced expression of *nkx2.5* following knockdown of either *scn5Laa* or *scn5lab* suggests a disruption of one or more of the molecular mechanisms that normally act to specify the cardiac cell fate during gastrulation.

Prior studies in zebrafish have demonstrated that *gata5* is a potent positive regulator of *nkx2.5* expression, potentially analogous to the role played by *gata4* or *gata6* in mammals.[15, 22] Notably, we found that *scn5Laa* regulates *nkx2.5* expression without affecting levels of *gata5*, analogous to *fgf8* signaling.[23] This suggests that *scn5Laa* may act downstream of *gata5* or *fgf8* or in a parallel, previously-undefined pathway to regulate cardiac development. Knockdown of *scn5Laa* and *scn5Lab* also resulted in changes in the expression of *gata4* and *hand2* in the anterior lateral mesoderm. In zebrafish, both *gata4* and *hand2* are expressed in larger domains of anterior lateral mesoderm than *nkx2.5* and are important for normal cardiogenesis.[14, 24, 25] Reduced *gata4* expression was observed to cause defects in cardiac chamber growth and looping, while loss of *hand2* resulted in embryos with significantly fewer embryonic cardiomyocytes.[24, 25] Moreover, the expression domain of *hand2* appears to more accurately mark the population of early myocardial progenitors in zebrafish than *nkx2.5*.[14] Rather than being required for the patterning or boundaries of the heart-forming region in mesoderm, *hand2* appears to have a direct, permissive role in promoting cardiac differentiation within this cell population.[14, 25] Taken together, the diminished number of cardiac progenitor cells observed in zebrafish cardiac sodium channel morphants is likely to be a consequence of the reduced expression level and/or domain of *nkx2.5*, *gata4*, and *hand2*, leading to a reduction in the size of the heart-forming region in anterior lateral mesoderm and limiting overall myocardial potential.

Voltage-gated sodium channels may contribute to early cardiogenesis independent of membrane electrophysiology

Supratherapeutic doses of TTX or other sodium channel active drugs did not perturb cardiac development. Cardiogenesis also proceeded normally when sodium was withdrawn from the external media in which embryos developed, another method for blocking inward I_{Na}. These findings thus suggest that zebrafish cardiac sodium channels may act in development via mechanisms that are independent of membrane depolarization.

Mounting evidence indicates that voltage-gated sodium channels subserve multiple functions whose disruption may underlie the phenotypes we describe. For example, sodium channel complexes play a role in cell adhesion.[26] Pore-forming Na_v1 sodium channel α subunits are known to interact with one or more ancillary β subunits (encoded by *SCN1B-4B*) that modulate α subunit expression and function.[1, 2] Through their extracellular immunoglobulin (IG) domains, β subunits also mediate interactions between the sodium channel complex and other cell adhesion molecules and extracellular matrix proteins.[26] Sodium channels are also known to interact with many other functionally-diverse proteins including 14-3-3, Nedd-4 type ubiquitinases, calmodulin, ankyrin G, syntrophins, plakophilin, and fibroblast growth factor homologous factors,[27, 28] so it is possible that the sodium channel α subunit itself could act as a scaffold to facilitate intracellular signaling by these associated proteins within the channel complex.

Alternatively, sodium channel α subunits may generate non-electrogenic intracellular signals via mechanisms that are intrinsic to the channel protein itself. The C-terminal tail of L-type calcium channels, for example, is cleaved from the pore-forming α subunit and translocates from the membrane to the nucleus where it regulates gene expression.[29] In this context, it is notable that zebrafish harboring a nonsense mutation in the cardiac L-type calcium channel α1c gene (*island beat*) display atrial arrhythmias and a small, non-contractile, hypoplastic ventricle by 72hpf.[30] By contrast, we found that pharmacological blockade of calcium entry through L-type calcium channels over a similar time period did not significantly affect ventricular development. Future studies are required for additional insight into the possible non-electrogenic functions of cardiac voltage-gated sodium and calcium channels.

Limitations

In these experiments, we cannot exclude the possibility that intracellular sodium conductance (e.g. channels acting on the membrane of an organelle rather than at the cell surface) is required for normal development. Second, rescue of morphant phenotypes with full length *scn5Laa* or *scn5Lab* mRNA has not been possible to date. This is likely due to inadequate translation of the 6kb transcripts, as we have been unable to detect an epitope tag engineered into the rescue construct. Finally, knockdown of either *scn5Laa* or *scn5Lab* resulted in a severe cardiac phenotype. It is possible that the two genes, which have significantly diverged in amino acid sequence, may subserve different functions during heart development that were not uncovered by our assays. Alternatively, it is possible that manipulating the expression of one gene altered the expression or function of the other.

In summary, the results presented here argue that sodium channels are required for heart development in addition to their canonical role as regulators of heart rhythm. Moreover, the data suggest that this developmental role is mediated by a non-electrogenic function of the channel.

Novelty and Significance

What is known?

- Activation of the Voltage-gated sodium channels leads to the depolarization of excitable membranes in mature myocardium, skeletal muscle and nerve.
- Mutations in *Scn5a*, the predominant cardiac sodium channel gene, are associated with multiple heritable disorders of heart rhythm.
- While S*cn5a* $^{+/-}$ *mi*ce develop myocardial fibrosis in senescence, *Scn5a* $^{-/-}$ mice develop marked abnormalities of ventricular morphogenesis by embryonic day 10.5, and die by day 11.5.

What new information does this article contribute?

- In zebrafish, *scn5a* orthologs are expressed in the gastrulating embryo, prior to the differentiation of excitable tissues.
- Antisense knockdown of either zebrafish cardiac sodium channel resulted in reduced expression of early markers of cardiomyocyte fate and marked chamber dysmorphogenesis.
- These findings suggest that the zebrafish cardiac sodium channels affect heart development via a non-electrogenic mechanism

Voltage-gated sodium channels are the principal determinants of membrane excitability. In the heart, mutations in the cardiac sodium channel gene *Scn5a* are well-described in heritable arrhythmia syndromes. While sodium channels play a critical role in the mature heart, the function of *Scn5a* during cardiac development is less well understood. Here we used zebrafish embryos to investigate the role of sodium channels in cardiogenesis. We observed that zebrafish cardiac sodium channel genes are expressed before myocardial differentiation. Embryos treated with sodium channel antisense displayed reduced expression of early cardiogenic transcription factors including *nkx2.5*, *gata4*, and *hand2*, leading to diminished numbers of cardiac progenitor cells, cardiac chamber dysmorphogenesis, and perturbed looping. Prolonged pharmacological blockade of sodium current failed to phenocopy channel knockdown, suggesting that sodium channels act in early development via mechanisms that are independent of membrane electrophysiology. These studies may contribute to an improved understanding of the spectrum of clinical phenotypes linked to mutations in *Scn5a*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Non-standard Abbreviations and Acronyms

ALPM	Anterior lateral plate mesoderm
ATX II	Anemone toxin
CHO	Chinese hamster ovary
DsRed	Discosoma sp. red fluorescent protein
E	embryonic day
GFP	green fluorescent protein
Hpf	hours post fertilization
$Na_v1.5$	sodium channel alpha subunit 1.5

nuc	nuclear
TTX	Tetrodotoxin

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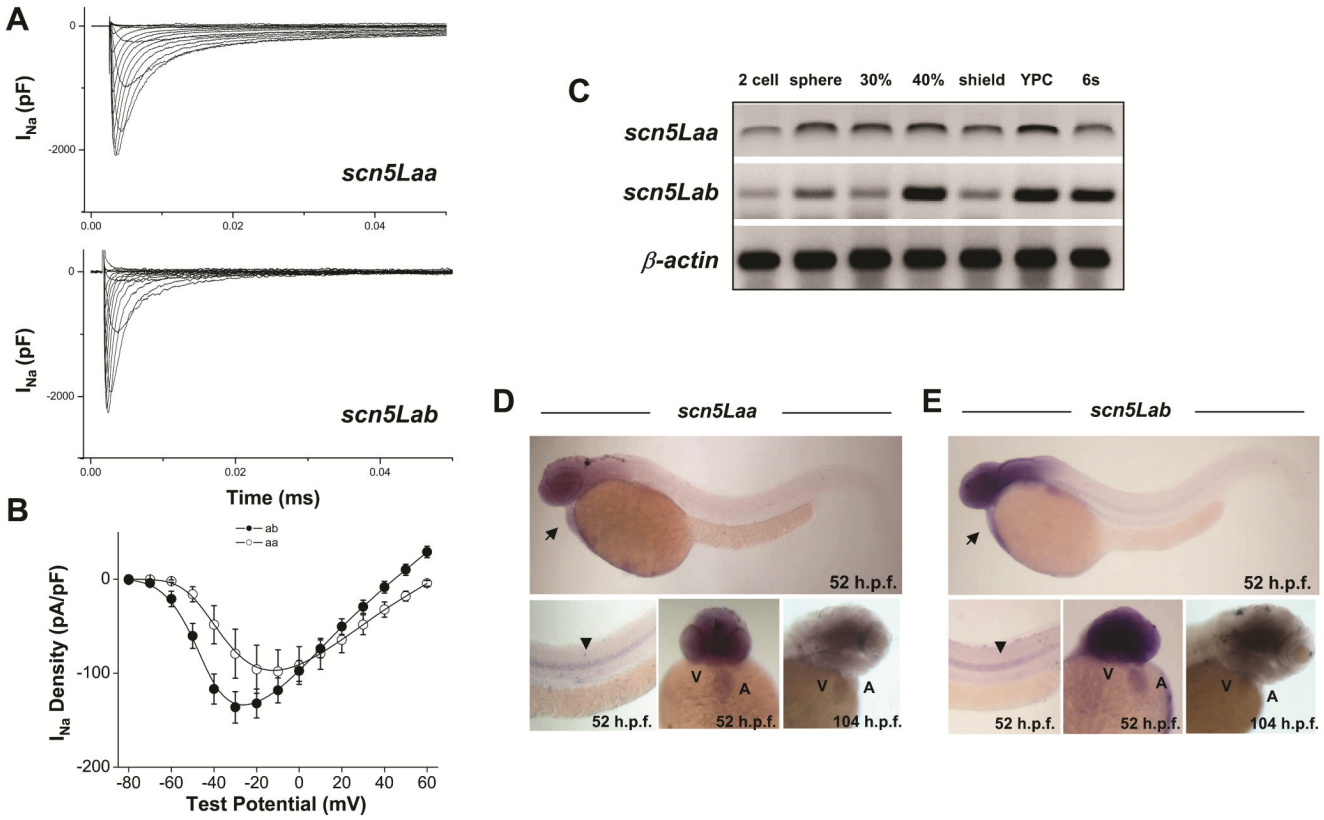

FIGURE 1. Function and developmental expression of zebrafish cardiac-type sodium channels

A, **B**. Whole cell sodium currents (A), and current-voltage relationships in CHO cells transfected with full-length *scn5Laa* and *scn5Lab* (B). **C**. Temporal expression of *scn5Laa* and *scn5Lab* in early embryos (0–12hpf) by RT-PCR. YPC = yoke plug closure stage, s = somites. **D, E**. By whole-mount *in situ* hybridization, sodium channel gene expression was detected diffusely in both heart chambers before displaying predominately ventricular expression by 104hpf. Transcripts were also detected in the brain and spinal cord. Developmental stages are as labeled. Arrow, heart. Arrowhead, spinal cord. A, atrium. V, ventricle.

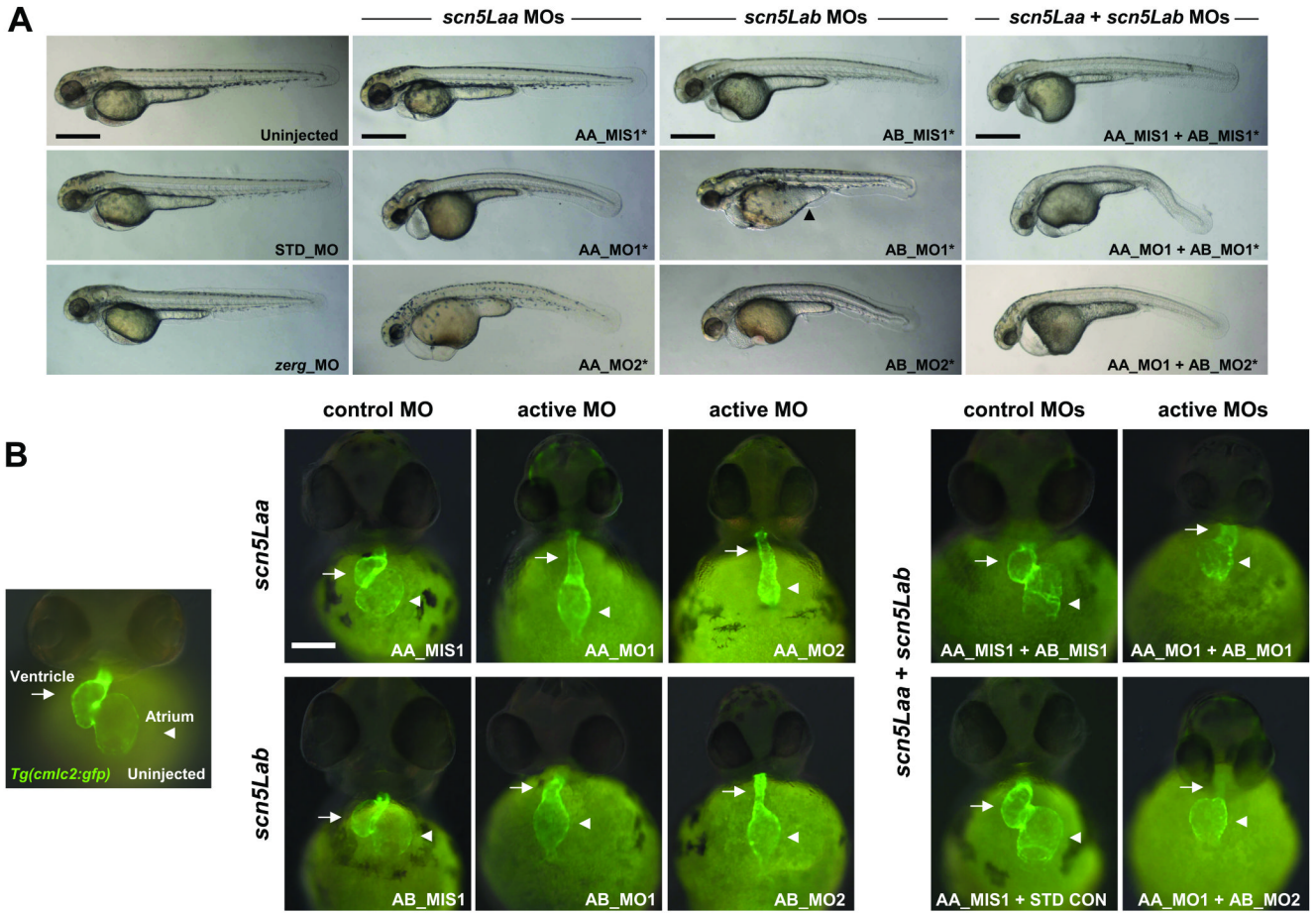

FIGURE 2. Zebrafish *scn5Laa* and *scn5Lab* are each required for normal cardiac development

A. Embryos at 58–62hpf following treatment with active or control antisense morpholinos. All embryos were also treated with the p53 morpholino. Injection of p53 morpholino alone did not cause any identifiable phenotype (Online Figure VII). Note that head size is reduced in all sodium channel morphants and that many embryos injected with AB_MO1 display a yoke sac extension abnormality (arrowhead). All scale bars = 500μM. **B**. Cardiac developmental defects in morphant *Tg(cmlc2:GFP)* embryos. AA_MO1 and AB_MO2 caused the most severe cardiac phenotypes at the lowest doses. Knockdown of both *scn5Laa* and *scn5Lab* resulted in cardiac defects that were more severe than knockdown of either sodium channel alone, particularly with respect to ventricular morphogenesis. Arrows = ventricle. Arrowheads = atrium. Scale bar = 150μM. Treatments are as labeled: STD_MO = standard control morpholino, *zerg*_MO = cardiac potassium channel morpholino, AA_MIS1 = *scn5Laa* 5-mismatch control morpholino, AA_MO1 = *scn5Laa* translation-blocking morpholino (ATG initiation site), AA_MO2 = second *scn5Laa* translation-blocking morpholino (5'UTR), AB_MIS1 = *scn5Lab* 5-mismatch control morpholino, AB_MO1 = *scn5Lab* splice-blocking morpholino (E6I6), AB_MO2 = second *scn5Lab* splice-blocking morpholino (E25I25).

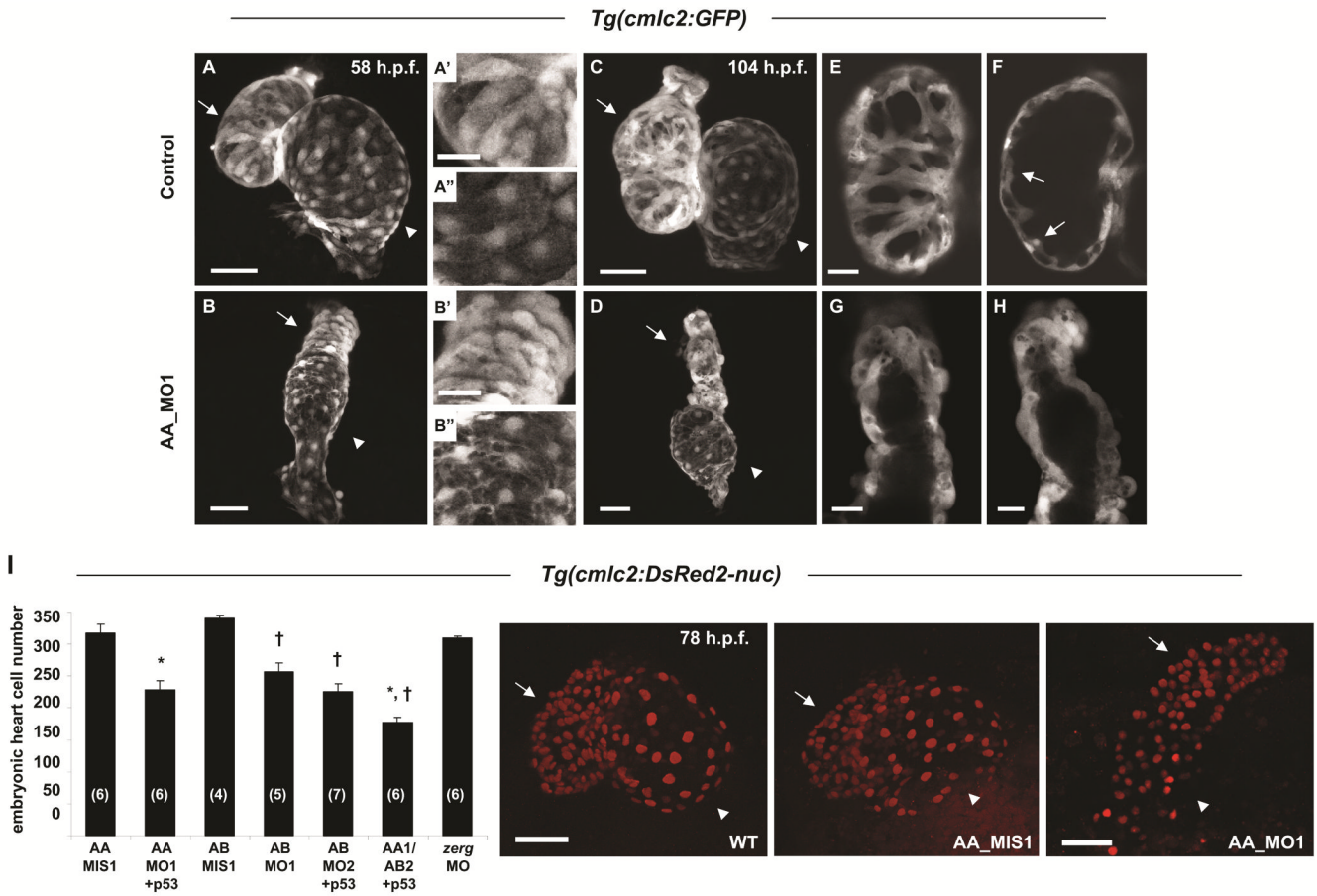

FIGURE 3. Sodium channel morphant embryos have reduced numbers of embryonic cardiomyocytes

A–H. Confocal reconstructions of hearts of control *Tg(cmlc2:GFP)* embryos (A, C) or clutchmates injected with the *scn5Laa* translation inhibitor morpholino (AA_MO1) (B, D) illustrate that sodium channels are required for normal numbers of embryonic cardiomyocytes. Arrows, ventricle and arrowheads, atrium at 58hpf (A, B) and 104hpf (C, D), respectively. Higher magnification of ventricular and atrial chambers presented in A', B' and A", B", respectively, illustrate similar chamber-specific cellular morphology in both control (A', A") and morphant (B', B") embryos. Scale bars = 50μm (A, B, C, D) and 20μm (A', A", B', B"). **E, F**. Serial confocal sections through the ventricular chamber of control embryos revealed trabeculation by 104hpf (arrows in F). Scale bar = 20μm. **G, H**. At the same timepoint, *scn5Laa* morphant ventricles remained a single layer (shown are ventricles from two different morphant embryos). Scale bars = 20μm. **I**. Confocal reconstructions of the embryonic heart in *Tg(cmlc2:DsRed2-nuc)* embryos permitted quantification of deficits of embryonic cardiomyocytes in morphant embryos at 60–62hpf. *Scn5Laa and scn5Lab* morphant hearts have significantly fewer embryonic cardiomyocytes than those of mismatch control morpholino-injected clutchmates and embryos injected with a morpholino targeting the *zerg* cardiac potassium channel. Results are mean ± s.e.m. (N) in bar graph = number of hearts analyzed. *, $P<0.01$ versus AA_MIS1 and †, $P<0.01$ versus AB_MIS1, ANOVA. Embryos injected with both AA_MO1 and AB_MO2 morpholinos (*scn5Laa, scn5Lab* double knockdown) had fewer cardiomyocytes than embryos injected with either AA_MO1 or AB_MO2 alone (177±8 versus 228±14 and 226±12, respectively) but the results were not statistically significant following ANOVA of all 7 groups. Where indicated, p53 morpholino

was co-injected with active morpholinos as a control for non-specific morpholino toxicity. Photos are representative wild type, *scn5Laa* control, and *scn5Laa* morphant embryo hearts at 78hpf. Arrows, ventricle. Arrowheads, atrium. Scale bars = 50μm.

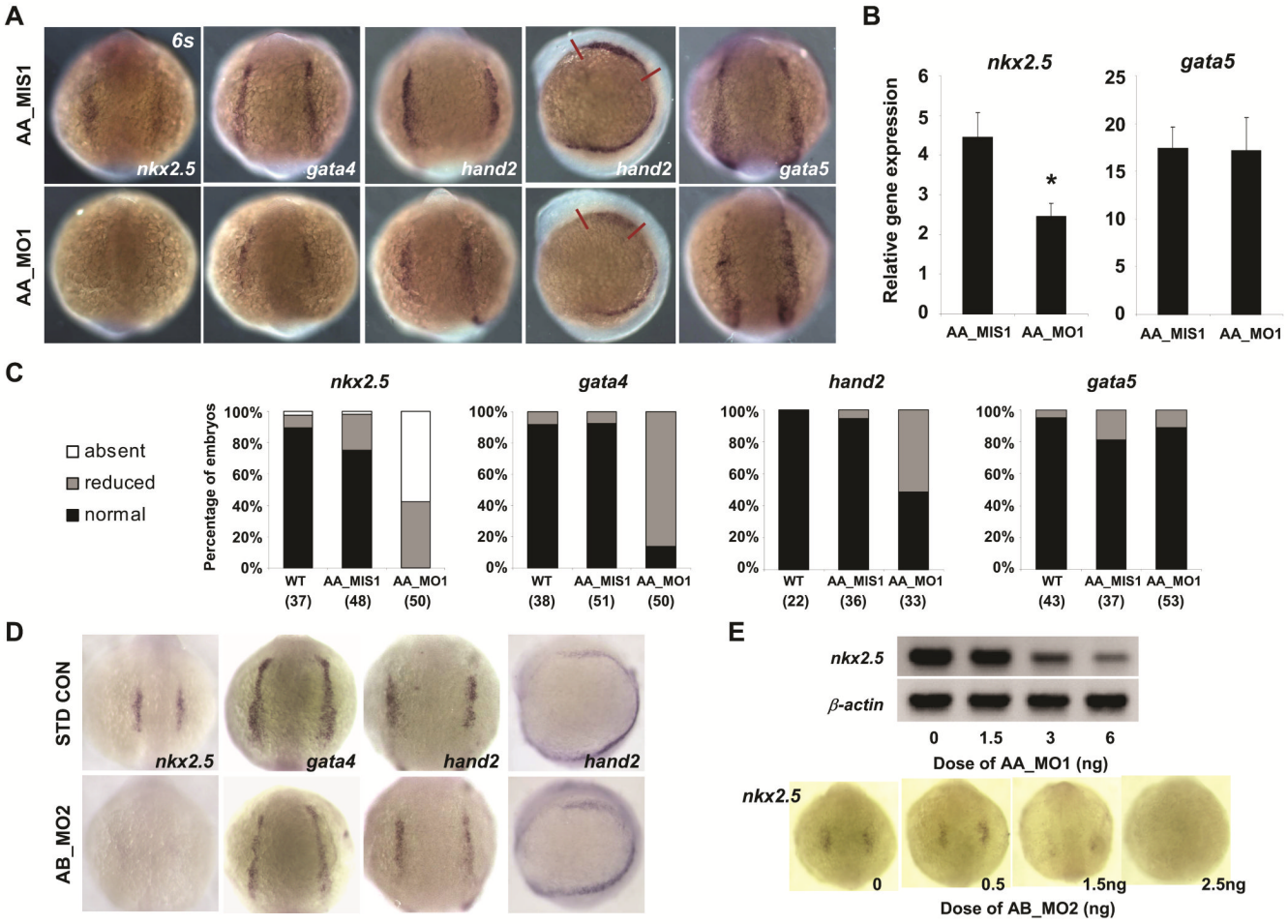

FIGURE 4. Sodium channel knockdown perturbs the expression of cardiac precursor genes in anterior lateral mesoderm

A. Analysis of gene expression in the early heart-forming region of anterior lateral plate mesoderm at the 6-somite stage by *in situ* hybridization. Embryos injected with *scn5Laa* translation inhibitor morpholino (AA_MO1) had reduced expression of *nkx2.5 gata4*, and *hand2* but no change in *gata5* expression compared to embryos injected with 5-mismatch control morpholino (AA_MIS1). All embryos shown are from the same injected clutch. Scale bar = 200μm. **B**. Real-time quantitative RT-PCR performed using independent clutches of embryos also revealed significantly reduced *nkx2.5* expression but no change in *gata5* expression following injection of AA_MO1 compared to injection of AA_MIS1 control morpholino. Results are mean ± s.e.m for three independent experiments, each performed in triplicate. $P<0.05$ for *nkx2.5*, T-test. **C**. Quantification of numbers (n) of *scn5Laa* morphant embryos displaying absent, reduced or normal (comparable to wild type) expression levels of cardiac precursor genes as assessed by *in situ* hybridization. **D**. Knockdown of *scn5Lab* also resulted in markedly-reduced expression of *nkx2.5* and *gata4* at 6 somites. However, no significant change in the expression of *hand2* was observed. **E**. Sodium channel morpholinos reduce *nkx2.5* expression in a dose-dependent manner as shown by RT-PCR (AA_MO1) and *in situ* hybridization at 6 somites (AB_MO2).

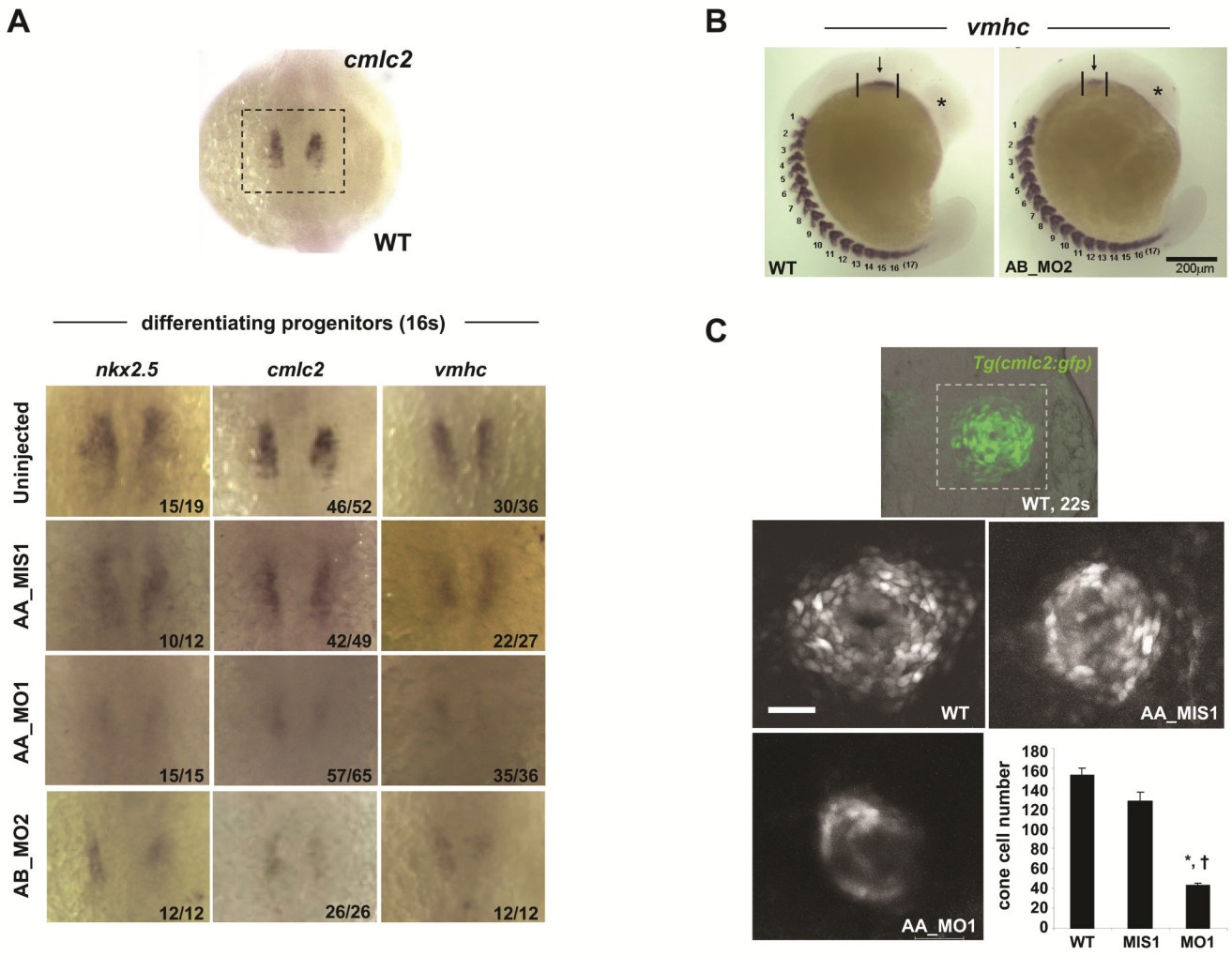

FIGURE 5. Sodium channel knockdown results in reduced numbers of differentiating cardiomyocytes

A. By *in situ* hybridization, *scn5Laa* and *scn5Lab* morphant embryos at the 16 somite stage display markedly reduced expression of *nkx2.5* and of the myocardial sarcomeric genes *cmlc2* and *vmhc* compared to control embryos. Treatment groups are as labeled. The number of embryos displaying the phenotype/the total number of embryos assessed is indicated in the lower right corner of each panel. **B**. Deficiencies in cardiac differentiation are not due to developmental delay. Shown is an embryo injected with the AB_MO2 morpholino compared to an uninjected clutchmate. Despite dramatic differences in the size of the heart field and head, both embryos have equivalent numbers of somites. **C**. Confocal reconstructions of the developing heart cone in *Tg(cmlc2:GFP)* embryos at the 22-somite stage (embryo in top panel is shown for orientation). Scale bar = 50μm. *Scn5Laa* morphant heart cones (AA_MO1, n=4) have significantly fewer differentiating cardiomyocytes than the heart cones of wild type (WT, n=6) and control-injected (AA_MIS1, n=3) clutchmates. Results are mean ± s.e.m. *, $P<0.01$ versus wild-type and †, $P<0.01$ versus AA_MIS1-injected, ANOVA.

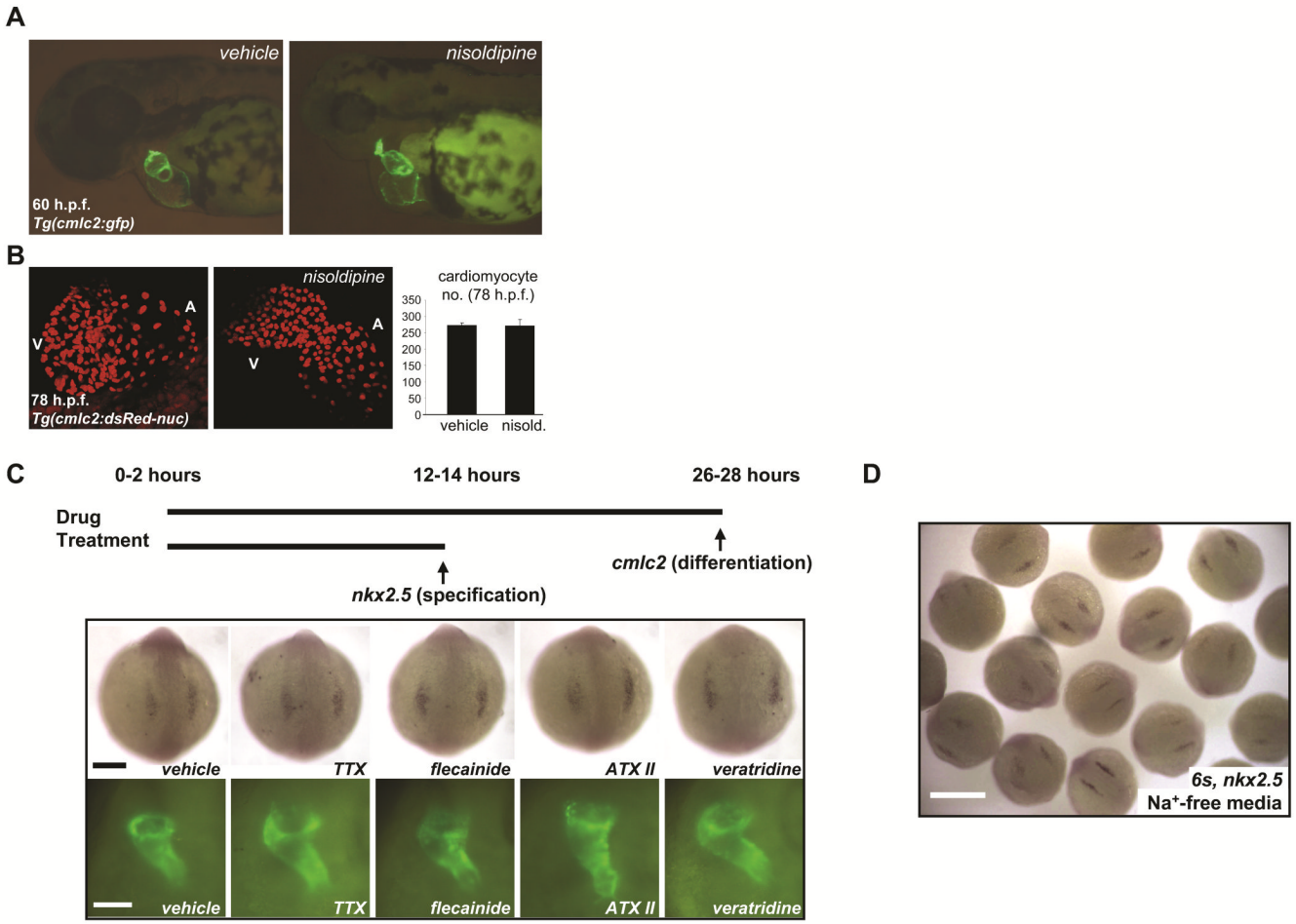

FIGURE 6. Voltage-gated sodium channels may regulate early cardiac development independent of membrane electrophysiology

A. Rearing of embryos in 10μM solution of the L-type calcium blocker nisoldipine inhibited heart-beating at all stages examined through 78hpf but did not perturb early chamber formation or the initiation of looping. **B**. Analysis of vehicle-treated and nisoldipine-treated *Tg (cmlc2:DsRed2-nuc)* embryos indicated that L-type calcium channel-blockade did not affect embryonic cardiomyocyte number through 78hpf. Results are mean ± s.e.m for vehicle-treated (n = 2) and nisoldipine-treated (n = 3) embryos, respectively. **C**. Prolonged exposure of developing embryos to pharmacological modulators of sodium channel function failed to disrupt either cardiac specification or differentiation. *In situ* hybridization for *nkx2.5* at the 6–8 somite stage (top) and fluorescent microscopy of the heart tubes of *Tg(cmlc2:GFP)* embryos at 26–28hpf (bottom), after the indicated treatments (see Table 2). TTX = tetrodotoxin, ATX II = anemone toxin II. Scale bars = 200μm (top) and 100μm (bottom). **D**. In situ hybridization for *nkx2.5* at the 6 somite stage after incubation from the 1 cell stage in sodium-free media, to inhibit inward sodium current (I_{Na}). Early heart development proceeded normally. Heart tubes also form normally (not shown). Scale bar = 500μm.

Table 1

Developmental effects of pharmacological modulators of ion channel function.

target	agent	type	delivery	[drug]	n	phenotypes
VGSC	tetrodotoxin	inhibitor	bath solution	500μm	74	• Normal heart tube formation (68/70). • Total neuromuscular paralysis (70/70)
			bath solution	500μm	26	• Normal *nkx2.5* expression (24/26).
			microinjection	1mM	99	• Normal heart tube formation (99/99). • Total neuromuscular paralysis (93/99).
VGSC	lidocaine	inhibitor	bath solution	500μm	200	• Normal heart tube formation (200/200). • Epiboly defects at higher doses.
VGSC	anemone toxin II	activator	bath solution	10μM	25	• Normal *nkx2.5* expression (20/25).
			microinjection	100μM	50	• Normal heart tube formation (50/50). • Convulsions, tonic contraction elicited with mechanical stimulation.
VGSC	veratridine	activator	bath solution	100μM	34	• Normal *nkx2.5* expression (26/34).
			microinjection	1mM	50	• Normal heart tube formation (50/50). • Convulsions, tonic contraction elicited with mechanical stimulation.
VGSC; ERG	flecainide	inhibitor	bath solution	400μm	46	• Normal heart tube formation (45/46). • Bradycardia, 2:1 heart block, less frequent silent ventricle or irregular atrial rhythm on day 2 in morphologically normal hearts, similar to ERG block alone (45/45).
			bath solution	400μm	29	• Normal *nkx2.5* expression (23/29).
LTCC	nisoldipine	inhibitor	bath solution	10μm	100	• Normal heart tube formation (100/100). • No heart beat, circulation at any stage. • Atrium, ventricle, outflow tract morphologically normal through day 2.5. • Cardiac looping normal through day 2.5.

target	agent	type	delivery	[drug]	n	phenotypes
						• Markedly decreased *GFP* intensity (~*cmlc2* promoter activity) at all stages.
LTCC/ TTCC	mibefradil	inhibitor	bath solution	250μm	77	• Normal heart tube formation (77/77). • Bradycardia, weak contractility on day 2 in morphologically normal hearts (77/77).
ERG	E-4031	inhibitor	bath solution	250μm	93	• Normal heart tube formation (93/93). • Arrhythmias on day 2 in morphologically normal hearts: – bradycardia, silent ventricle (85/93) – irregular atrial rhythm, silent ventricle (7/93) – silent heart (1/93)

VGSC = voltage-gated sodium channel, LTCC = L-type calcium channel, TTCC = T-type calcium channel, ERG = ether-a-go-go related gene potassium channel. For bath immersion, embryos were dechorionated and placed in drug solutions by the 64 cell stage. For microinjection, 3 nanoliters of drug solution were injected in 2 different locations of the yoke sac of 1–4 cell stage embryos. Cardiac lineage specification assessed by *in situ* hybridization for *nkx2.5* at 8 somites. Differentiation assessed by heart tube formation and heart beating in *Tg(cmlc2:GFP)* embryos at 28–30 h.p.f. See methods for additional details.

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