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Reduced sodium channel function unmasks residual embryonic slow conduction in the adult right ventricular outflow tract

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Abstract

Rationale: In patients with Brugada syndrome, arrhythmias typically originate in the right ventricular outflow tract (RVOT). The RVOT develops from the slowly conducting embryonic outflow tract.

Objective: We hypothesize that this embryonic phenotype is maintained in the fetal and adult RVOT and leads to conduction slowing, especially after sodium current reduction.

Methods and Results: We determined expression patterns in the embryonic myocardium, and performed activation mapping in fetal and adult hearts, including hearts from adult mice heterozygous for a mutation associated with Brugada syndrome (Scn5a1798insD/+). The embryonic RVOT was characterized by expression of Tbx2, a repressor of differentiation, and absence of expression of both Hey2, a ventricular transcription factor, and Gja1, encoding the principal gap-junction subunit for ventricular fast conduction. Also, conduction velocity was lower in the RVOT than in the right ventricular free wall. Later in development, Gja1 and Scn5a expression remained lower in the subepicardial myocardium of the RVOT than in RV myocardium. Nevertheless, conduction velocity in the adult RVOT was similar to that of the right ventricular free wall. However, in hearts of Scn5a1798insD/+ mice and in normal hearts treated with ajmaline, conduction was slower in the RVOT than in the right ventricular wall.

Conclusions: The slowly conducting embryonic phenotype is maintained in the fetal and adult RVOT, and is unmasked when cardiac sodium channel function is reduced.
role in cardiac remodeling in both pressure overload as well as ischemia models of cardiac injury (reviewed by [335 Goumans, MJ (2008)], [336 Dobaczewski, M (2011)]). TGFβ activates a pro-fibrotic program involving the upregulation of extracellular matrix proteins and myofibroblast differentiation in a SMAD3-dependent manner (reviewed by [336 Dobaczewski, M (2011)]). The latter points to Fstl1 as a candidate mediator for TGFβ signaling. However, in contrast to the profibrotic TGFβ response, Fstl1 is observed to have an overall protective role in different models of myocardial stress, either by stimulating neo-vascularization in infarcted areas or by protecting cardiomyocytes from apoptosis in trans-aortic constriction (TAC) induced cardiomyopathy. [174 Shimano, M (2011)], [165 Ogura, Y (2012)], [167 Oshima, Y (2008)], [157 Liu, S (2010)], [156 Liu, S (2006)], [168 Ouchi, N (2010)].

In addition to structural remodeling, also electrical remodeling occurs after myocardial stress. Electrical remodeling encompasses the change in conduction and excitation-contraction coupling of cardiomyocytes, which is thought to cause arrhythmias in post injury hearts (reviewed by [337 Cutler, MJ (2011)]). Changes in ion currents and concentrations lie at the basis of the electrical remodeling in heart failure [338 Coronel, R (2013)]. Interestingly, TGFβ1 stimulation of isolated cardiomyocytes results in changes in ion currents (reviewed by [339 Ramos-Mondragon, R (2008)]). We hypothesize that TGFβ might influence electrical remodeling in the post injured heart via activation of Fstl1 which in turn effects conduction, possibly via activation of the Na/K-ATPase.

We have demonstrated a novel signaling role for the endocardium-derived connective tissue, which via the TGFβ regulated expression of Fstl1 influences AV conduction.

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Introduction

The right ventricular outflow tract (RVOT) is the main origin of arrhythmias in the Brugada syndrome. [340 Morita, H (2003)] The mechanism underlying arrhythmias in Brugada syndrome is debated but most likely involves conduction delay or block in the presence of subtle structural discontinuities. [341 Coronel, R (2005)] This arrhythmogenic substrate is modulated by variations or mutations in ion channels and other genes. [342 Berne, P (2012)] The electrocardiographic signs and arrhythmias often only become evident after application of sodium channel blockers. [343 Bayes de, LA (2012)] Why arrhythmias in patients with Brugada syndrome preferentially originate in the RVOT is unclear. During development, the RVOT forms from the embryonic outflow tract. [22 De la Cruz, MV (1977)] The embryonic outflow tract is a slowly conducting structure with low expression levels of connexin43 (Cx43), Cx40, and of the cardiac sodium channel protein alpha subunit (Scn5a). [28 Moorman, AFM (2003)], [44 de Jong F. (1992)] Low expression of Scn5a and/or Cx43 reduce the safety of propagation, also known as conduction reserve, and increase the susceptibility for arrhythmias. [344 van Rijen, HVM (2004)] We hypothesize that the adult RVOT myocardium retains aspects of this embryonic outflow tract phenotype and that these embryonic aspects contribute to lower conduction reserve in the RVOT. We investigated the expression pattern of genes associated with the embryonic outflow tract phenotype and with conduction in the mouse RVOT, in relation to conduction velocity, during development and in the adult heart. Furthermore, we investigated the functional consequences of reduced sodium current on RVOT conduction by pharmacological sodium channel blockade and in a mouse model with a human cardiac sodium channel mutation associated with the Brugada syndrome.
Methods

Transgenic mice
The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) or the European Commission Directive 2010/63/EU and was approved by the institutional review board. Tbx2cre transgenic mice and Scn5a1798insD/+ mice have been described previously. [18 Aanhaanen, WT (2009)], [345 Remme, CA (2009)] In this study we used three E14.5 and E17.5 and adult FVB/N wildtype mice. Furthermore, we used five E14.5 Tbx2cre/cre mice and five littermate controls, and five adult Scn5a1798insD/+ (129P2-OlaHsd strain) mice and three littermate controls.

Immunohistochemistry and in situ hybridization
Mice were stunned by inhalation of CO and killed by cervical dislocation, after which the adult heart or the embryos were collected. The adult heart or and whole embryos were fixed in 4% PBS buffered formaldehyde, embedded in paraplast® and sectioned at 7-8 μm for immunohistochemistry and at 10-14 μm for RNA in situ hybridization. RNA in situ hybridization was performed according to a previously described method. [346 Moorman, AFM (2001)] Probes for selected genes have been described previously. [18 Aanhaanen, WT (2009)] For immunohistochemistry, rehydration, unmasking, blocking and washing steps were performed according to the protocol of the tetramethylrhodamide based amplification kit (Perkin Elmer). Primary antibodies used for mouse sections were: cTnI rabbit polyclonal (1:250; Hytest Ltd); Tbx3 goat polyclonal (1:500; Santa Cruz Biotechnology); Cx40 mouse monoclonal (1:250; US Biological); Cx43 mouse monoclonal (1:250; BD Transduction). Secondary antibodies when using amplification were: Biotinylated donkey-anti-goat (1:250; Jackson Immunology); biotinylated goat-anti-rabbit (1:250; DAKO); biotinylated goat-anti-mouse (1:250; DAKO). For visualization without the amplification step, secondary antibodies coupled to an Alexa fluorescent (1:250; Invitrogen) were used.

Western blot
Using a Ultraturrax, protein extracts were made in a RIPA buffer (50mM Tris-HCl pH8, 150mM NaCl, 1% NP-40, 0.2% sodium deoxycholate,0.1% SDS containing 1mM NaVO3, 1mM DTT, 1mM PMSF) in the presence of a protease inhibitor cocktail tablet (Roche Complete mini 11836170001). Protein concentrations were determined using the Protein Assay Reagent BCA kit (Thermo Scientific 23227) using their specified protocol. Laemmli sample buffer (for 5x concentrated 0.3 M Tris-HCl pH 6.8, 10% SDS, 50% glycerol, 25% 2-mercaptoethanol, 0.02% bromphenol blue) was added to the protein lysates and the samples were heated to 95°C for 5 min, then chilled on ice and briefly centrifuged. For Cx43 and Nav1.5 westernblots 2 and 20 μg of protein samples were loaded on a 10%
and 6% acrylamide denaturizing gel, respectively. After electrophoresis the proteins were transferred to Immobilon-PSQ transfer membrane (Milipore ISEQ09120) using a trans-blot semi dry transfer cell (Biorad 170-3940) machine. The membranes were then sliced in two parts, one part for the Cx43 or Nav1.5, the other part for the loading control Calnexin.

The membranes were washed with TBST buffer (Tris 50mM NaCl150mM 0.1% Tween) and blocked with TBST containing 2.5% milk powder (Protifar Pus Interpharm 634) for 1 hour at room temperature. Primary antibodies were diluted in blocking buffer in the following concentrations: α-Calnexin(VWR 208880) 1:5000, α-Cx43(Milipore AB1727) 1:1000, α-NAV1.5(Sigma-Aldrich S0819) 1:500. Membranes were incubated with the primary antibodies over night at 4°C. Secondary antibody anti Rabbit HRP(GE Healthcare Life Sciences NA9340) was diluted 1:2500 in blocking buffer and incubated for 2 hours at room temperature. The blots were then visualized using the chemiluminescent ECL-PLUS western blot reagens (Amersham RPN2132) and a ImageQuant LAS 4000 biomolecular imager analyzer. For quantification of the western blot bands the AIDA (raytest Isotopenmeßgeräte GmbH v4.26.038) image analyzer program was used.

**Preparation of the hearts and recording of optical action potentials**

**Adult hearts**

Mice were stunned by inhalation of CO and killed by cervical dislocation, after which the heart was excised, cannulated, mounted on a Langendorff perfusion set-up, and perfused at 37°C with Tyrode’s solution (in mmol/L) 128 NaCl, 4.7 KCl, 1.45 CaCl₂, 0.6 MgCl₂, 27 NaHCO₃, 0.4 Na₂HPO₄, and 11 glucose (pH maintained at 7.4 by equilibration with a mixture of 95% O₂ and 5% CO₂). The hearts were placed in 10 ml Tyrode’s solution containing 15 μM Di-4 ANEPPS and subsequently in an optical mapping setup. Excitation light was provided by a 5 Watt power LED (filtered 510 +/- 20 nm). Fluorescence (filtered > 610 nm) was transmitted through a tandem lens system on CMOS sensor (100 x 100 elements, MICAM Ultima). Activation patterns were measured during sinus rhythm and ventricular pacing at a basic cycle length of 120 ms (twice the diastolic stimulation threshold). We defined transverse fiber direction by the slowest conduction velocity. Conduction was slowed by perfusion with 2 µM Ajmaline (Giluritmal®, Carinopharm). Conduction velocity was measured after 8 min administration of Ajmaline.

**Fetal hearts**

The hearts where removed from the fetus and incubated for 5 minutes with Tyrode’s solution containing 5 µM Di-4 ANEPPS at 37°C. After incubation, fetal hearts were superfused with Tyrode’s solution and placed on the stage of an inverted microscope set-up for recording optical signals. The size of the adult heart and the intrinsic heterogeneity of the outflow
tract myocardium did not allow measurement of conduction velocity following central stimulation. Therefore, we analyzed isochronal patterns during sinus rhythm instead.

**Quantitative PCR**
RNA was isolated from dissected hearts using the NucleoSpin® RNA II (Marchery Nagel Cat:740955.50). From 1 μg of RNA, single stranded cDNA was made using SuperScript™ II Reverse Transcriptase (Invitrogen Cat: 18064-071) with Oligo(dT) primers, according to manufacture’s protocol. Quantitative PCR (qPCR) was performed using the LightCycler® 480 (Roche) and the LightCycler® 480 SYBR Green I Master solution (Roche Cat: 04887352001). Primer sequences for the gene products Scn5a, Cx43, Tnni3 and Hprt are available on request. qPCR data was analyzed using LinRegPCR program. [279 Ruijter, JM (2012)]

**Analysis and Statistics**
Optical action potentials were analyzed using custom-made software based on MATLAB R2006b (MathsWorks Inc., Natick, MA). [347 Potse, M (2002)] The local moment of activation was defined as the maximum positive dV/dt of the action potential. Group comparisons were performed using (repeated) ANOVA after, if necessary, factor correction. Values are given as mean +/- SEM. A P-value of 0.05 was considered statistically significant.
Results and Discussion

The working myocardial gene program in the RVOT is established just before birth

The embryonic outflow tract is a slowly conducting structure marked by the expression of Tbx2, and absence of Gja1 and Hey2 (hairy/enhancer-of-split related with YRPW motif 2). [348 Fischer, A (2003)], [28 Moorman, AFM (2003)] To investigate whether this embryonic signature is maintained in the RVOT, we investigated the expression patterns of these markers in the early fetal (E14.5) and late fetal (E17.5) mouse hearts by in situ hybridization. At E14.5, the expression of Gja1 was absent from the RVOT myocardium but present in the right ventricle (Figure 1). This pattern was similar to that of Hey2. The expression of Tbx2 was maintained in the RVOT, in a pattern complementary to Gja1 and Hey2 (arrows in Figure 1A). At stage E17.5, Gja1 and Hey2 were still absent from the RVOT region that had relatively decreased in size compared to the ventricle. In contrast to stage E14.5, however, the expression of Tbx2 was absent from the RVOT myocardium marked by Tnni3 (Figure 1B). Scn5a, unlike Gja1, was already expressed in the outflow tract at E12.5 (Online Figure IA) and remained expressed in the RVOT throughout later stages. These data indicate that the fetal RVOT, at least in part, retains the signature of the embryonic outflow tract. The absence of Tbx2 expression just before birth (E17.5) suggests that the myocardium in the RVOT has acquired aspects of the ventricular working myocardial phenotype.

In the adult heart, the expression of Gja1 was lower in the subepicardial region of the RVOT myocardium compared to the right or left ventricular myocardium (Figure 1C and Online Figure IB), and the expression of Tbx2 and Hey2 was absent from the ventricular myocardium, resembling stage E17.5. We measured Gja1 and Scn5a mRNA levels by quantitative RT-PCR in the entire adult RVOT. For this purpose, the RVOT was defined as the smooth walled myocardium located at the base of the right ventricle below the pulmonary valves (Figure 2A). Tnni3 was used as a marker of myocardial tissue and was not quantitatively different between the RVOT and the right and left ventricle (data not shown). The expression levels of both Gja1 and Scn5a were significantly lower in the RVOT than in the RV and LV, but not between LV and RV (Figure 2B/C). Next we measured the protein levels of CX43 and NAV1.5 by Western blot, and found that they were lower in the RVOT than in the RV (Figure 2D/E). These data demonstrate that, also in the adult RVOT, aspects of the embryonic program are maintained. The samples likely contained a mixture of both the subendo- and subepicardium of the RVOT and therefore the quantifications probably represents an underestimation of the differences.
Figure 1.
In the fetal heart the RVOT is composed of slowly conducting myocardium. Panel A and B show in-situ hybridizations in E14.5 (A), E17.5 (B) and adult (C) wildtype hearts of expression of Tnni3, Gja1, Tbx2 and Hey2 in the right ventricle and RVOT (n=3 for all stages). Note that Gja1 expression is not present in the RVOT. The black bar indicates 0.1 mm. TP, truncus pulmonalis; RV, right ventricle; RVOT, right ventricular outflow tract; VS, ventricular septum; AO, aorta.
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Figure 2.
The expression of Gja1 and Scn5a is low in the RVOT. Panel A illustrates the part of the heart that was defined as RVOT. The bar graphs in panel B and C show respectively the expression levels (qPCR, corrected for Hprt and Tnni3) of Gja1 and Scn5a in the RVOT and right and left ventricle (N=5). Panel D and E show the protein levels of CX43 (n=6) and NAV1.5 (n=3) in the RVOT and right and left ventricle. Calnexin was used as a loading control. Abbreviations as in figure 1.

Slow conduction in the fetal OFT

In the fetal heart (E14.5), conduction was slower in RVOT than in the RV (Figure 3), consistent with the absence of Gja1 in the RVOT. Because Tbx2 represses non-working myocardium genes including Gja1 [349 Hoogaars, WMH (2007)], we studied hearts from homozygous Tbx2 mutant mice. The RVOT of these mice was normal and Gja1 expression was not different from wild type mice (Figure 3A). Furthermore, conduction in the RVOT of wild-type mice and Tbx2 mutants was not different (Figure 3B). These data imply that RVOT conduction characteristics are not regulated by Tbx2. Tbx3, the related transcriptional regulator and functional homologue of Tbx2, is not likely to compensate for loss of Tbx2, as it is not expressed in the myocardial component of the outflow tract or RVOT during development or in the adult (Online Figure IB). [349 Hoogaars, WMH (2007)]
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Figure 3.
Panel A shows in-situ hybridizations of E14.5 Tbx2cre/cre heart showing the expression of Tnni3, Gja1 and cre in the right ventricle and RVOT (n=3). Panel B shows a reconstructed activation pattern of the right ventricle and the RVOT in an E14.5 wildtype heart. The straight lines connect the two pairs of pixels that were used for the calculation of conduction velocity. The bar graph in panel C shows the average conduction velocity in the right ventricle and RVOT. Panel D shows the activation patterns during stimulation at a site in the LV, RV and RVOT in the adult heart. The bar graph in panel E shows the average longitudinal and transversal conduction velocity in the adult right and left ventricle and the RVOT (n=3). In the RVOT longitudinal conduction velocity could not be measured. Abbreviations as in figure 1.

Slow conduction is not maintained in the adult RVOT
The transversal conduction velocity recorded following pacing in the adult RVOT was not slower than in the right or left ventricle (Figure 3D/E). We did not quantify longitudinal conduction velocity in the RVOT because the typically anisotropic activation pattern following stimulation was not present. We argued that lower levels of GJA1 and NAV1.5 in the RVOT would not lead to conduction slowing because the safety for conduction is very high due to the non-linear relation between electrical coupling and conduction velocity, consistent with the observation that in healthy humans no arrhythmias originate in the RVOT. [350 Jongsm, HJ (2000)], [351 Van Rijen, HV (2004)], [352 Hoffman, BF (1957)] However, we hypothesized that an additional reduction of sodium current would be required to lead...
to further reduction in safety of conduction and thereby unmask the intrinsic regional
difference in conduction reserve. For that reason, we measured conduction velocity in
the RVOT and RV before and after administration of the sodium channel blocker ajmaline
(Figure 4A). Ajmaline reduced the transversal conduction velocity more in the RVOT than
in the RV (Figure 4B/C).

**Figure 4.**
Conduction velocity is slowed more in the RVOT than in the RV after reduction of the sodium current. Panel A/D show reconstructed activation patterns of the RV (right) and RVOT (left) during central stimulation in wildtype hearts before and after administration of ajmaline (A) or in wildtype and Scn5a1798insD/+ hearts (D). Panel B/E show the transversal conduction velocity in the RV and RVOT in the absence and presence ajmaline (B) or in wildtype and Scn5a1798insD/+ mice (E). Panel C/F shows the difference between conduction velocity in the right ventricle and RVOT in the presence and absence of ajmaline (C) or in wildtype and Scn5a1798insD/+ mice (F). Abbreviations as in figure 1.

**Sodium channel dysfunction results in conduction slowing in the RVOT**
We then measured ventricular conduction in mice with a 1798InsD mutation in Scn5a,
the mouse equivalent of the human 1795InsD mutation found in patients with Brugada
syndrome. [353 Remme, CA (2006)] During sinus rhythm, crowding of isochrones indicated that conduction was delayed in the right ventricle and RVOT of *Scn5a*^{1798insD/+} mice compared to wildtype litter mates (Online Figure II). We subsequently measured conduction velocity following stimulation from the RV and RVOT. In *Scn5a*^{1798insD/+} mice, conduction velocity was lower in both the RV and RVOT than in wild type mice (Figure 4D). Conduction slowing was significantly more pronounced in the RVOT than in the RV of *Scn5a*^{1798insD/+} mice (Figure 4E/F). Thus, reduced sodium channel function unmasked slow conduction in the adult RVOT, indicating maintenance of the embryonic gene program responsible for slow conduction.

We have recently proposed a unifying mechanism for arrhythmogenesis in Brugada syndrome patients [341 Coronel, R (2005)] involving subtle structural discontinuities in the RVOT myocardium. This hypothesis does not offer an explanation for the preferential location of these abnormalities in the RVOT since these structural abnormalities and a mutation are also present in the LV and RV. [354 Hoogendijk, MG (2010)] Our data indicate that in the RVOT the expression of Gja1 and Scn5a is persistently lower than in the RV which offers an explanation for the preference of conduction delays or block in the RVOT, and not the RV, of Brugada syndrome patients, especially after pharmacological or genetically reduced sodium channel function.

**Limitations**

In this study we used mouse hearts which are electrophysiologically different from human hearts. This may complicate extrapolation from our findings in mice to man. Furthermore, we determined changes in transcript and protein abundance which do not predict with certainty whether there will be changes in the presence of functional channels. However, our functional data support the notion that differences in expression between the RVOT and the RV underlies the electrophysiological differences between these components. An genetic rescue experiment in order to remove the electrophysiological differences between the RVOT and RV could further support our hypothesis.

**Conclusion**

We demonstrate that the Tbx2-positive, Gja1-negative myocardial gene program and the slow conducting phenotype of the embryonic outflow tract myocardium are maintained in the RVOT until birth. In the adult heart, lower expression levels of Gja1 and Scn5a are still observed in the RVOT. We demonstrate that this is associated with less conduction reserve in the RVOT than in the right ventricle, resulting in more pronounced conduction slowing in the RVOT than in the right ventricle when sodium current is decreased. Our data provide an explanation why conduction delay occurs preferentially in the RVOT, as is observed in patients with Brugada syndrome. [341 Coronel, R (2005)]
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Online Figure I.

Panel A shows an immuno-staining for CTNI (right) and NAV1.5 (left) in the OFT at E12.5. Panel B shows at the right in situ hybridizations for Tnni3 and Tbx3 and at the left an immuno-staining for TBX3, CTNI and nuclei. Abbreviations as in figure 1.
Online Figure II.
Panel A shows a reconstructed activation pattern during sinus rhythm in the hearts of a wildtype (upper panel) and Scn5a<sup>S1798insD</sup>/+ (lower panel) mouse. Abbreviations as in figure 1.