Arrhythmic substrate, slowed propagation and increased dispersion in conduction direction in the right ventricular outflow tract of murine Scn5a+/- hearts

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Abstract

Aim: To test a hypothesis attributing arrhythmia in Brugada Syndrome to right ventricular (RV) outflow tract (RVOT) conduction abnormalities arising from Na+ channel insufficiency and fibrotic change.

Methods: Arrhythmic properties of Langendorff-perfused Scn5a+/− and wild-type mouse hearts were correlated with ventricular effective refractory periods (VERPs), multi-electrode array (MEA) measurements of action potential (AP) conduction velocities and dispersions in conduction direction (CD), Na+1.5 expression levels, and fibrotic change, as measured at the RVOT and RV. Two-way ANOVA was used to test for both independent and interacting effects of anatomical region and genotype on these parameters.

Results: Scn5a+/− hearts showed greater arrhythmic frequencies during programmed electrical stimulation at the RVOT but not the RV. The Scn5a+/− genotype caused an independent increase of VERP regardless of whether the recording site was the RVOT or RV. Effective AP conduction velocities (CV†), derived from fitting regression planes to arrays of observed local activation times were reduced in Scn5a+/− hearts and at the RVOT independently. AP conduction velocity magnitudes derived by averaging MEA results from local vector analyses, CV*, were reduced by the Scn5a+/− genotype alone. In contrast, dispersions in conduction direction, were greater in the RVOT than the RV, when the atrioventricular node was used as the pacing site. The observed reductions in Na+1.5 expression were attributable to Scn5a+/−, whereas increased levels of fibrosis were associated with the RVOT.

Conclusions: The Scn5a+/− RVOT recapitulates clinical findings of increased arrhythmogenicity through reduced CV† reflecting reduced CV* attributable to reduced Na+1.5 expression and increased CD attributable to fibrosis.

Keywords Brugada Syndrome, conduction velocity, Na+ channel, right ventricular outflow tract.

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The Brugada Syndrome (BrS) is characterized by an increased risk of arrhythmogenic sudden cardiac death particularly in middle aged (approx. 40–45 years) males (Brugada et al. 2002, Priori et al. 2002, Antzelevitch et al. 2005, Eckardt et al. 2005, Probst et al. 2010). It has been thought to be primarily a right ventricular (RV) disease (Antzelevitch et al. 2005). It is often associated with electrocardiographical (ECG), right preaortic ST segment elevation (Brugada & Brugada 1992, Brugada et al. 1998), right bundle branch block and ventricular tachycardia (VT) or fibrillation (VF) originating from the RV outflow tract (RVOT) (Nagase et al. 2002, Haissaguerre et al. 2003, Kofune et al. 2011). However, the detailed pathophysiology producing re-entrant waves of excitation remains uncertain. A repolarization disorder was suggested on the basis of the reduction in Na⁺ current (I_{Na}) in relationship to transient outward K⁺ currents (I_{to}). This would shorten RV epicardial action potential durations (APD) and thereby produce a transmural dispersion of RV action potential waveform (Kurita et al. 2002, Antzelevitch et al. 2007). Alternatively, reduced I_{Na} would be expected to slow action potential (AP) conduction particularly in the RVOT (Nagase et al. 2002, Lambiase et al. 2009) assuming that its slowly conducting embryonic phenotype persists into the adult (Boukens et al. 2013). The RVOT has thus been implicated in both the electrocardiographical abnormalities and the delayed epicardial conduction associated with potentially fatal ventricular arrhythmia in BrS (Berruezo et al. 2004, Morita et al. 2008) in a depolarization disorder hypothesis (Kasanuki et al. 1997, Tukkie et al. 2004, Coronel et al. 2005, Meregalli et al. 2005, Postema et al. 2008).

Ventricular fibrillation in patients with BrS can often be induced at the RVOT (Morita et al. 2003). This might relate to structural abnormalities in the RVOT in BrS, detectable as wall motion abnormalities (Takagi et al. 2001) and fibrotic or fatty endocardial infiltration (Papavassiliu et al. 2004). Some clinical studies have also explored for electrophysiological abnormalities localized to the RVOT. However, such studies in human subjects were confined to non-invasive assessments of RVOT conduction delays employing body surface mapping (Izumida et al. 2004), signal-averaged ECGs (Hisamatsu et al. 2004) or tissue Doppler echocardiography (Tukkie et al. 2004). Nevertheless, there have been a limited number of invasive human studies. These demonstrated endocardial RVOT activation delays (Kanda et al. 2002) as well as electrogram prolongation and steeper activation recovery intervals (ARI) and restitution curves in RVOT endocardial recordings (Lambiase et al. 2009).

However, clinical studies investigating epicardial heterogeneities in depolarization and repolarization are difficult to perform. One report described epicardial electrograms from the conus branch of the right coronary artery running over the RVOT surface. This demonstrated activation delays not found in the endocardium (Nagase et al. 2002), as well as a shortening of ARIs in the RVOT using an epicardial catheter in the great cardiac vein during ST elevation. Another study demonstrated epicardial, but not endocardial spike-and-dome AP waveforms during open chest surgery (Kurita et al. 2002). An explanted BrS heart in which VF was induced by programmed stimulation, showed a subendocardial rather than subepicardial re-entrant activation, with RV activation slowing in an absence of transmural repolarization gradients in the RVOT. Its RV contained abundant fibrous and adipose tissue (Coronel et al. 2005). A further explanted BrS heart combined ST segment elevation with a reduced local activation in basal RV subepicardium. This showed a fibrosis and fatty infiltration that in combination with altered I_{Na} and consequently I_{Cal} and I_{to} could produce a current-to-load mismatch at its junction with more normal myocardial tissue. This could account for both reduced conduction velocities and the characteristic BrS ECG pattern (Hoogendijk et al. 2010a,b). Such a suggestion was compatible with results from experimental and modelling studies (Hoogendijk et al. 2011).

Of experimental models for BrS, the RV pharmacological wedge preparation precludes localization of arrhythmias in the RVOT. However, an in vivo, closed chest canine study demonstrated ECG changes characteristic of BrS following cooling of a small epicardial RVOT region, and produced a ‘spike and dome’ appearance in the monophasic AP (MAP) in the epicardium. However, this model did not reproduce the loss of the AP dome (Nishida et al. 2004). Experimental systems containing genetic modifications directly replicating those known to exist in BrS may provide more specific models for the associated physiological abnormalities whilst permitting studies impractical in human subjects. Up to 30% of BrS patients have SCN5A mutations involving the cardiac voltage-gated Na⁺ channel α-subunit (Na,1.5) (Chen et al. 1998, Antzelevitch et al. 2007, London et al. 2007). A murine, Na,1.5 haploinsufficient, heterozygotic, Sca5a+/-, model (Papadatos et al. 2002) has proven useful in physiological studies of both atrial and ventricular arrhythmic properties in BrS (Lei et al. 2005, Stokoe et al. 2007, Hao et al. 2011). Sca5a+/- hearts show a 50% reduction in RV Na,1.5 expression and in I_{Na} (Martin et al. 2012). Loss of function SCN5A mutations are associated with a complex range of phenotypes that include sinus node dysfunction and progressive conduction disorders in both clinical situations (Wilde & Coronel 2008) and in the mouse model.
(Papadatos et al. 2002). The Scn5a+/− mouse reproduces many key features of human BrS. It shows ECG ST elevation (Martin et al. 2010b) and ventricular arrhythmogenesis accentuated by flecainide and relieved by quinidine (Martin et al. 2010a,b), particularly in the right ventricular (RV) epicardium (Matthews et al. 2013) with delayed RV response latencies and increased transmural repolarization gradients (Martin et al. 2010b). It also shows an age-dependent RV fibrosis (van Veen et al. 2005, Jeevaratnam et al. 2010) in line with clinical findings (Coronel et al. 2005).

The present study first explored whether murine Scn5a+/− hearts replicated the arrhythmic properties of RV and RVOT sites observed in human BrS under conditions of extrasystolic intraluminal stimulation. The experiments then related these arrhythmic properties to differences between their electrophysiological features and those of WT hearts. These included ventricular effective refractory periods (VERPs), conduction velocities and dispersions in conduction direction (CD). These physiological findings were next compared with differences in Na,1.5 expression and tissue fibrosis. Differences in all these parameters were then statistically correlated with either the genotype or the site at which the parameter was measured.

Materials and methods

All procedures were performed in conformity with good scientific practice recommendations (Persson 2013, Zhang et al. 2013). The studies described here were based on a total of 23 WT and 21 Scn5a+/− hearts from inbred 129sv mice aged 3–8 months.

Arrhythogenic properties of isolated Langendorff-perfused hearts

The electrophysiological experiments employed isolated Langendorff-perfused hearts, prepared and studied under conditions and solutions described on earlier occasions (Matthews et al. 2013, Zhang et al. 2013). Hearts were first studied at their intrinsic rates without applied stimulation. Endocardial pacing was then delivered using a 1-Fr bipolar mouse pacing catheter (NuMED; Hopkinton, NY) inserted into the RV through the pulmonary artery. This delivered stimulation first to the endocardium of the RVOT free wall <2 mm below the pulmonary artery, and then as close to the RV apex as could be reached by the catheter (Fig. 1a). This stimulation order was reversed in half the hearts studied. First, regular, 8 Hz, pacing stimuli...
used 2-ms square-wave pulses whose amplitude was adjusted to twice the excitation threshold (DS2A iso-
lated constant voltage stimulator; Digitimer, Welwyn
Garden City, UK). This was followed by a programmed
electrical stimulation (PES) procedure. This began by
imposing standard 8 Hz baseline-pacing stimuli for
20 s. The subsequent drive trains consisted of cycles of
eight paced beats (S1) each followed by an extrastimu-
lus (S2) initially imposed at an S1–S2 interval equal to
the pacing interval, then reduced by 1 ms with each
subsequent cycle until the VERP was reached.

Our strategy of endocardial stimulation permitted
measurement of electrical signals using an epicardial
monophasic action potential (MAP) recording electrode
(Hugo Sachs, Harvard Apparatus, Edenbridge, Kent,
UK) successively placed against the RVOT and the RV
free wall. The MAPs were pre-amplified with a
NL100AK head stage, band-pass filtered (0.5 Hz–
1 kHz; Neurolog NL 125/6 filter; Digitimer, Welwyn
Garden City, Hertfordshire, UK) and digitized at a sam-
ping frequency of 5 kHz with a micro 1401plus MKII
laboratory interface (Cambridge Electronic Design,
Cambridge, UK). Analysis of MAP waveforms was per-
formed using Spike II software (Cambridge Electronic
Design).

**Multi-electrode array recordings and quantification of
effective epicardial conduction properties**

WT and Scn5a+/− hearts (Fig. 1a,b) were subject to
regular (8 Hz) pacing applied at (a) the endocardium
using the 1-Fr bipolar pacing catheter inserted through
a left atrial incision to access the atroventricular node
(AVN) and at (b, c) the epicardium of the LV (b) base
and (c) apex using a custom-made suction electrode.

Epicardial AP recordings were made using 32 channel
MEAs (ME32-FAI-System; Scientifica, Uckfield, UK)
applied to the RVOT and RV free walls simultaneously.

Each MEA contained 32 electrodes of 50 µm diameter in a
1.5 × 1.5 mm configuration with a 300-µm inter-
electrode distance (Fig. 1c), sampling at 10 kHz per
channel. The potential at each recording site was
recorded against a reference level provided by a single
electrode placed remote from the recording array on the
metal cannula through which the heart was perfused.
This therefore provided monophasic recordings of the
voltages generated at each recording site.

Previous studies have pointed out that the intrinsic
deflection reflecting the AP upstroke corresponds to
the negative going deflection in monophasic recordings of
excitable activity (Dower 1962, Janse & Rosen
2006). Off-line determinations of local activation
times (LATs) at each recording site were related to
timings of the first detected waveform in the recording
array at which the waveforms showed their maximal
negative slopes. Such an approach agreed with deter-
minations using their peak deflections.

These LATs were first used to derive effective epi-
cardial conduction velocities (CVt), by fitting a regres-
sion plane to the LATs (King et al. 2013). The LATs
were first visualized on three-dimensional plots to
locate the largest area forming a uniform plane for
optimization. Results were discarded if uniform areas
covering at least 24 adjacent channels were not found:
this would not be consistent with stable conduction
velocities. However, very few (<5 of approx. 100) car-
diac cycle recordings studied for each heart were thus
excluded. In approximately half of the analyses, the
entire array could be used in regression analysis. This
gave multiple-τ values between 0.90 and 0.98 corro-
borating the approximation procedure. The resulting
CVt values, reflecting the reciprocal of the gradient of
the best-fit plane, were expressed in dimensions of
velocity (m s−1).

A local vector analysis then analysed the LATs in
successive, neighbouring 2 by 2 square sets of four
recording points successively labelled from L1 to L4
along the perimeter of each set. Row and column time
difference vectors were then calculated from their cor-
responding LATs as \( t^*_x = ((L1 + L2) − (L3 + L4))/2 \)
and \( t^*_y = ((L1 + L4) − (L2 + L3))/2 \) respectively. These
in turn provided the magnitude, \( d(\sqrt{(t^*_x)^2 + (t^*_y)^2}) \)
and direction, \( \theta \), of their contained velocity vector. A
mean conduction velocity magnitude, CVt, could then
be obtained by averaging the magnitude values from the
entire array. The dispersion in CD, was obtained from
the standard deviation of the corresponding \( \theta \)
values. Coherent conduction paths with low CD would
give CVt values close to the corresponding CV*. Curved or
incoherent conduction paths with high CDs would give
CVt values less than the CV*.

**Western blot and histology**

Age, litter and sex-matched hearts of further 6-month
old WT and Scn5a+/− mice were excised for either
Western blot or histological anatomical studies. Wes-
tern blot analysis was used to estimate Na,1,5 protein
expression in the RV and RVOT of both Scn5a+/− and
WT hearts. Total protein was extracted from the RV
and RVOT strip using lysis buffer containing (mM) 20
Tris–HCl (pH 8.0), 137 NaCl, 2 EDTA and 1% Triton
x-100 and 10% glycerol. The protein concentration
were normalized to the same concentration. Ten micro-
gram of protein extract was separated by 4–8% SDS–
PAGE gels, and transferred to polyvinylidene difluoride
membranes (Immuno-blot PVDF Membrane; Bio-Rad).

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The membrane was blocked in 5% non-fat dried milk in PBST (phosphate-buffered saline (PBS), 0.05% (v/v) Tween 20 (Sigma-Aldrich, Gillingham, Dorset, UK), pH 7.4), and then probed with an anti-human Na,+1.5 antibody, followed by a horseradish peroxidase-conjugated secondary antibody. The protein signal was visualized using the ECL Western blotting detection kit and Hyperfilm ECL (Amersham Biosciences, Bath, Avon, UK). The specimen was re-probed using an anti-GAP-DH antibody (Chemicon, Watford, UK), which served as loading control. The anatomical studies used isolated hearts flushed with Krebs-Henseleit buffer (Matthews et al. 2013, Zhang et al. 2013) and perfused with 4% buffered formalin for 5 min before overnight formalin fixation. Gross transverse sections from apex to base then underwent routine tissue processing and paraffin embedding. The base and middle heart blocks were sectioned at a 7 μm thickness towards the RVOT and RV for picrosirius red staining (Sigma-Aldrich), viewing, magnification and digital recording using the Nano Zoomer 2.0 Digital Pathology system (Hamamatsu, UK). Quantification of fibrosis was performed for each heart on four randomly selected photomicrographs representing sections spaced by a minimum distance of 14 μm.

Statistical analysis

Incidences of arrhythmia were analysed using Fisher’s exact tests applicable to such categorical data. The remaining, continuous data, expressed as means ± SEM, were first tested using two-way ANOVA for correlated samples analysis of variance (SPSS; IBM, Portsmouth, Hamps., UK and GRAHPAD PRISM V6.1; La Jolla, CA, USA). Each two-way ANOVA tested for effects of the categorical groups representing genotype (i.e. either WT or Scn5a+/-) or choice of recording site (i.e. either RV or RVOT) upon the continuous data obtained from the experimental measurements. Such measurements yielded values of VERP, CV’, CV* and CD, and characterized Na,+1.5 expression and fibrotic change. This made it possible to demonstrate significant differences in these variates, and assess whether these differences could be attributed to independent or interacting effects of either genotype or anatomical site. Particular differences were then investigated by Bonferroni-corrected Student’s unpaired t-tests to a significance level of \( P < 0.05 \).

Results

Programmed electrical stimulation demonstrates greatest arrhythmogenicity at the Scn5a+/- RVOT

The experiments first explored arrhythmic properties during intrinsic activity, regular endocardial pacing and PES. An occurrence of VT was defined as a period containing three or more consecutive ectopic beats detected by epicardial recording. Neither WT nor Scn5a+/- showed spontaneous arrhythmic activity during 15 min runs of intrinsic activity or 2 min runs of regular pacing. In contrast, application of two successive runs of PES provoked arrhythmia with greater frequency in Scn5a+/- compared to WT hearts. This took the form of either sustained (Fig. 2a) or short VT episodes (Fig. 2b). Categorical data comparing incidences of arrhythmia in Scn5a+/- and WT hearts with pacing at either the RVOT or RV was analysed by Fisher’s exact test. With RVOT pacing, Scn5a+/- hearts showed greater arrhythmic incidences than WT: VT was then observed in 1 of 9 WT but 6 of 7 of Scn5a+/- hearts \((P < 0.01)\). In contrast, with pacing at the remaining RV, WT and Scn5a+/- hearts showed indistinguishable incidences of VT (2 out of 7 vs. 4 of 6 hearts respectively: \( P > 0.05 \)). Figure 2c,d plot the number of individual hearts (vertical axis) that showed arrhythmic incidents at particular S1S2 intervals (horizontal axis) during the PES protocol. They compare observations resulting from stimulation applied to the RVOT (c) and RV (d) of WT and Scn5a+/- hearts respectively. The asterisks indicate significant differences in the occurrence of arrhythmia between the Scn5a+/- and the WT. This representation demonstrated that more Scn5a+/- than WT hearts showed arrhythmia. For both RVOT and RV stimulation sites, when arrhythmia occurred, it appeared at longer S1S2 intervals in the Scn5a+/- than in the WT. Finally, with RV stimulation, fewer hearts showed arrhythmia. However, when arrhythmia did occur, it took place at longer S1S2 intervals than was the case for the RVOT. In conclusion, arrhythmic tendency was increased specifically with RVOT as opposed to RV stimulation in Scn5a+/- but not WT hearts.

Ventricular effective refractory periods

The arrhythmic properties outlined above were then compared with the observed differences in electrophysiological, anatomical and molecular properties. The latter were in turn correlated with independent or interacting effects from the Scn5a+/- or WT genotype, or recording from the RVOT or RV. Table 1 summarizes the detailed results of this analysis. VERPs could be obtained from PES runs that culminated in refractoriness as opposed to arrhythmia. The Scn5a+/- genotype exerted an independent effect of increasing VERP regardless of recording site (Table 1). VERPs were greater in the RVOTs of the Scn5a+/- than in the corresponding WT (WT: \( n = 17, 42.65 \pm 2.60 \) ms; Scn5a+/- \( n = 5, 70.6 \pm 6.24 \) ms; \( P < 0.01 \)). They
Figure 2 (a, b) VT episodes recorded from the free wall of the right ventricular outflow tract (RVOT) and right ventricle (RV) during programmed electrical stimulation (PES) of Scn5a+/− hearts. (a) Sustained episodes of VT in the RVOT of a Scn5a+/− heart. (b) Short episodes of VT in the RV of a Scn5a+/− heart. (c) and (d) summarize numbers of hearts showing arrhythmic episodes (vertical axis) at each progressively shortened S1S2 interval in the course of PES procedures (horizontal axis) applied to the Scn5a+/− (filled bar) and WT (open bar) during pacing at the RVOT (c) and RV (d). *P < 0.05; **P < 0.01.
were indistinguishable between the RVs of Scn5a+/- and WT hearts \( (P > 0.05; \text{WT}, n = 17, 43.36 \pm 4.07 \text{ ms}; \text{Scn5a+/-}, n = 5, 54.29 \pm 6.57 \text{ ms}) \).

**Table 1** Results of two-way ANOVA testing for independent or interacting effects of genotype and anatomical region on electro-physiological properties, Na,1.5 expression and fibrotic properties*

| Effect of genotype \( (\text{Scn5a+/- vs. WT}) \) | Effect of anatomical region \( (\text{RVOT vs. RV}) \) | Presence/absence of interaction | Number \((n)\) of Scn5a+/- hearts | Number \((n)\) of WT hearts |
|---|---|---|---|---|
| VERP | \( P < 0.001 \) (Scn5a+/- > WT) | \( P > 0.05 \) (NS) | \( P > 0.05 \) (NS) | 5 | 17 |
| Conduction velocity from planar fit to LATs (CV†) | | | | |
| AVN pacing site | \( P < 0.01 \) (Scn5a+/- < WT) | \( P < 0.01 \) (NS) | \( P > 0.05 \) (NS) | 17 | 16 |
| LV base pacing site | \( P < 0.01 \) (Scn5a+/- < WT) | \( P < 0.01 \) (NS) | \( P > 0.05 \) (NS) | 10 | 7 |
| LV apex pacing site | \( P < 0.01 \) (Scn5a+/- < WT) | \( P < 0.01 \) (NS) | \( P > 0.05 \) (NS) | 11 | 6 |
| Conduction velocity from local vector analysis of LATs (CV*) | | | | |
| AVN pacing site | \( P < 0.001 \) (Scn5a+/- < WT) | \( P > 0.05 \) (NS) | \( P > 0.05 \) (NS) | 17 | 14 |
| LV base pacing site | \( P < 0.0001 \) (Scn5a+/- < WT) | \( P < 0.01 \) (NS) | \( P > 0.05 \) (NS) | 9 | 7 |
| LV apex pacing site | \( P < 0.001 \) (Scn5a+/- < WT) | \( P > 0.05 \) (NS) | \( P > 0.05 \) (NS) | 12 | 6 |
| Conduction dispersion from local vector analysis of LATs (CD) | | | | |
| AVN pacing site | \( P > 0.05 \) (NS) | \( P < 0.001 \) (NS) | \( P > 0.05 \) (NS) | 17 | 14 |
| LV base pacing site | \( P > 0.05 \) (NS) | \( P > 0.05 \) (NS) | \( P > 0.05 \) (NS) | 9 | 7 |
| LV apex pacing site | \( P > 0.05 \) (NS) | \( P > 0.05 \) (NS) | \( P > 0.05 \) (NS) | 12 | 6 |
| Western blot: Na,1.5 expression | \( P < 0.01 \) (Scn5a+/- < WT) | \( P > 0.05 \) (NS) | \( P > 0.05 \) (NS) | 5 | 5 |
| Levels of fibrosis | \( P > 0.05 \) (NS) | \( P < 0.001 \) (NS) | \( P > 0.05 \) (NS) | 4 | 4 |

AVN, atrioventricular node; RV, right ventricular; RVOT, right ventricular outflow tract; LV, left ventricular; LATs, local activation times.

*Pacing at the RVOT resulted in higher incidences of arrhythmia in Scn5a+/- than WT hearts (Fisher’s exact test: \( P < 0.01 \)); Pacing at the RV yielded indistinguishable incidences between WT and Scn5a+/-.

**Effective conduction velocities**

 Epicardial MEA measurements comparing ventricular APs in RVOTs and RVs of WT and Scn5a+/- hearts were then made under regular AVN pacing that would be expected to initiate an excitation sequence approximating that expected in vivo. Results were compared with findings from stimulation at the LV base and apex. Figure 3A,B exemplifies MEA measurements at the RVOT (A) and RV of WT (B) hearts during regular AVN pacing. It shows (a) traces obtained from each array element and (b) false colour maps representing their times to first peak. (c) The resulting plots of LATs (vertical axis) at different positions in the array (x and z horizontal axes) typically showed smooth surfaces suggesting a graded activation of the epicardial surface. It was therefore possible to derive an effective conduction velocity, CV†, by a least-squares fit of a regression plane through this array of LATs. Figure 4a summarizes the results of these CV† determinations (means ± SEMs) from runs obtained from the RVOTs and RVs of WT and Scn5a+/- hearts under each pacing condition.

With pacing at the AVN, LV base and LV apex, both the Scn5a+/- genotype and the choice of RVOT as recording site independently produced reductions in CV† (see Table 1 for results of two-way ANOVA). Pairwise testing then demonstrated that the CV† in the RVOT of the Scn5a+/- was reduced compared to that of the RVOT of WT. In addition, with pacing at the AVN and the LV base, the RVOT showed reduced CV†s compared with the RV in the Scn5a+/- and the WT respectively. Finally, with pacing at the LV base and apex, CV† was reduced in the RV of the Scn5a+/- relative to that in the RV of the WT (Fig. 4a; see legend for detailed results of pairwise testing).

**Conduction velocity magnitudes and directions from local vector analysis**

The effective conduction velocities determined above are the consequence of both the magnitude, and the direction, \( \theta \), of AP conduction through the MEA. These component variables were calculated for successive square arrays of four MEA recording sites (see Methods). The magnitude determinations were then...
averaged over all the determinations made for the array to provide a conduction velocity magnitude, \( CV^* \), whose means ± SEMs are shown in Figure 4b. The \( Scn5a^{+/0} \) genotype resulted in a reduced \( CV^* \) whether hearts were paced at the AVN, LV base or LV apex. In contrast, choice of RVOT or RV recording site exerted no influence on \( CV^* \) except with pacing at the LV base (See Table 1 for results of two-way ANOVA). Pairwise testing demonstrated that \( CV^* \) was reduced in the RVOT compared with the RV in the \( Scn5a^{+/0} \) with pacing at the AVN and in the WT with pacing at the LV base. \( CV^* \) was also lower in the RVOT of the \( Scn5a^{+/0} \) than the RVOT of the WT with AVN, LV base and LV apex pacing. Finally, there was a reduced \( CV^* \) in the \( Scn5a^{+/0} \) RV compared with the WT RV (Fig. 4b; see legend for detailed results of pairwise testing).

It was also possible to obtain estimates of the dispersion in CD, through the MEA recording sites. Figure 5A exemplifies velocity vector maps for the RVOT (Fig. 5Aa,c) and RV (b, d) of WT (a, b) and \( Scn5a^{+/0} \) (c, d) hearts. Each vector represents the magnitude and direction, \( \theta \), for each velocity at different positions within the MEA. The dispersion of CD, was then determined from the standard deviations of the values of \( \theta \) obtained through the MEA (means ± SEMs). CD was greater at the RVOT as opposed to RV, with pacing at the AVN, but not at either the LV base or apex. There was no influence from or interaction with genotype (see Table 1 for results of two-way ANOVA). Pairwise testing revealed that with AVN pacing, the \( Scn5a^{+/0} \) RVOT showed greater dispersions than either the RV of the WT or the RV of the \( Scn5a^{+/0} \). Similarly, the WT RVOT showed greater dispersions than the WT RV (Fig. 5Ba; see legend for detailed results of pairwise testing).

Na,1.5 expression and histological properties

The final experiments related the above physiological results to biochemical measures of Na,1.5 expression and histological measures of fibrotic change in the RVOT and RV of WT and \( Scn5a^{+/0} \) hearts [Fig. 6Aa, B(a-d)]. Na,1.5 expression levels were reduced in association with \( Scn5a^{+/0} \) genotype independent of whether these were measured in the RVOT or RV (see Table 1 for results of two-way ANOVA). Pairwise tests showed that Na,1.5 expression levels (Fig. 6Ab) in both the RV of the \( Scn5a^{+/0} \), and the RVOT of the \( Scn5a^{+/0} \) were lower than in the RV of the WT. In
contrast, differences in picrosirius red staining (Fig. 6Ba–d) demonstrated a greater level of fibrosis in the RVOT than the RV free wall regardless of genotype (see Table 1 for results of two-way ANOVA). Pairwise tests then demonstrated greater fibrosis in the RVOT than in the RV free wall in Scn5a+/−/C0 hearts (Fig. 6Be, see figure legend for detailed results of analysis).

Discussion

The BrS is an arrhythmic condition clinically characterized by the occurrence of potentially fatal arrhythmic episodes beyond middle age. Up to 30% of BrS patients have SCN5A mutations involving Na+1.5 (Chen et al. 1998, Antzelevitch et al. 2007, London et al. 2007). Patients with BrS were originally considered to have normal cardiac structure and function, but several studies report progressive myocardial structural abnormalities (Bezzina et al. 1999, 2003, Coromel et al. 2005, Remme et al. 2008, Persson & Persson 2012). Clinical evidence additionally implicates specific anatomical regions, particularly the RVOT, as the site for initiation of arrhythmias with the resulting regional differences in epicardial conduction velocity between the RVOT and RV then triggering epicardial re-entrant excitation waves (Meregalli et al. 2005).

Genetically modified mice have proven useful models for observed changes in cardiac arrhythmic disease, particularly BrS (Martin et al. 2012, Speerschneider & Thomsen 2013). Both Scn5a-1798insD (Stein et al. 2009) and Scn5a+/− mice carrying loss of function Scn5a mutations (Papadatos et al. 2002) show conduction defects. They also show a decreased Na+ channel function as well as progressive fibrotic invasion between the cardiomyocytes and altered gap junctions (van Veen et al. 2005, Stein et al. 2009). Both conduction and histological abnormalities were more prominent in the right than the left side of the heart in parallel with the arrhythmic substrate in BrS (Coromel et al. 2005, Postema et al. 2008, Jeevaratnam 2012).
et al. 2010, 2012, Lodder & Bezzina 2013). However, the related clinical, molecular and biophysical information related to these changes, including the extent to which these changes are involved in disease progression and arrhythmogenesis remain limited (Remme et al. 2008).

The present investigations use the Scn5a+/− model to explore for the existence and contributions of such factors to its arrhythmic properties. Previous studies demonstrated that the Scn5a+/− model (Papadatos et al. 2002) reproduces atrial and ventricular arrhythmia (Lei et al. 2005, Stokoe et al. 2007, Hao et al. 2011), electrophysiological (Martin et al. 2012), pharmacological (Martin et al. 2010a,b) and age-dependent fibrotic, properties (Coronel et al. 2005, van Veen et al. 2005, Jeevaratnam et al. 2010, 2012).

Figure 5 (A) Mappings of conduction directions from recordings made at the free walls of the right ventricular outflow tract (RVOT) and right ventricle (RV) of WT and Scn5a+/− hearts. Typical findings from WT (a, b) and Scn5a+/− (c, d) with recording from RVOT (a, c) and RV (b, d). (B) Determinations of the dispersion (CD) in conduction direction at the RVOT (left pair of histograms) and RV (right pair of histograms) for both WT (clear bars) and Scn5a+/− hearts (filled bars) sorted by the atrioventricular node (AVN) (a), LV base (b) and LV apex (c) pacing sites. CD: dispersion in conduction direction. With AVN pacing (Ba), the Scn5a+/− RVOT showed greater dispersions than either the RV of the WT (P < 0.01) or the RV of the Scn5a+/− (P < 0.01). Similarly, the WT RVOT showed greater dispersions than the WT RV (P < 0.01) (Ba). **P < 0.01.
associated with human BrS. The present experiments demonstrated and explored RVOT involvement in the arrhythmogenesis observed in isolated perfused whole hearts. They explored for correlations between anatomical regions and genotype, and arrhythmogenesis. They then further correlated these with conduction differences assessed by MEA recording, Na\textsubscript{v}1.5 expression and regional fibrotic change. Findings concerning each of these parameters were then statistically assessed using two-way ANOVA for the presence or absence of either independent or interacting effects of the \textit{Scn5a}\textsuperscript{+/–} vs. the WT genotype, and the RVOT or RV site at which the parameter was measured.

The electrophysiological experiments demonstrated (1) increased arrhythmic tendency with RVOT as opposed to RV stimulation in \textit{Scn5a}\textsuperscript{+/–}, but not WT hearts. This recapitulated clinical studies similarly implicating the RVOT in ventricular arrhythmia in BrS (Morita \textit{et al.} 2003), and previous reports that arrhythmic tendency varies with pacing site (Osadchii 2012). However, (2) VERPs were increased with the \textit{Scn5a}\textsuperscript{+/–} genotype regardless of RVOT or RV recording site. VERPs were greater in the RVOTs of \textit{Scn5a}\textsuperscript{+/–} than in corresponding WT but were indistinguishable between \textit{Scn5a}\textsuperscript{+/–} and WT RVs. These findings contrast with expectations of reduced or unchanged VERPs, predicted by hypotheses invoking Phase II re-entry phenomena arising from altered relative magnitudes of \textit{I}_\text{Na} and \textit{I}_{to}, in arrhythmia in BrS (Lukas & Antzelevitch 1996, Antzelevitch 2006, Veeraraghavan \textit{et al.} 2013). Under conditions of constant velocity, increased VERP would also tend to increase AP wavelength, \( \lambda \), classically defined as the product \( \lambda = CV\uparrow \) VERP in the \textit{Scn5a}\textsuperscript{+/–} whose arrhythmic substrate would normally

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure6}
\caption{Assessments of Na\textsubscript{v}1.5 expression (A) showing western blot results (a) and their quantification (b) and fibrosis (B) showing typical histological results for WT (a, b) and \textit{Scn5a}\textsuperscript{+/–} (c, d) from the right ventricular outflow tract (RVOT) (a, c) and right ventricle (RV) (b, d), with their quantification (Be). Pairwise testing demonstrated that Na\textsubscript{v}1.5 expression levels (Ab) in both the RV of the \textit{Scn5a}\textsuperscript{+/–} (\( P < 0.05 \)), and the RVOT of the \textit{Scn5a}\textsuperscript{+/–} were lower than in the RV of the WT (\( P < 0.05 \)). It also demonstrated a greater fibrosis, assessed by picrosirius red staining, in the RVOT than in the RV free wall in \textit{Scn5a}\textsuperscript{+/–} hearts (\( P < 0.05 \)) (Be). ** \( P < 0.05 \).
\end{figure}
be expected to accompany a decreased $\lambda$. In contrast, (3) both the Scn5a+/– genotype and recording from the RVOT as opposed to RV independently resulted in reduced effective CV† determined by a planar fit to LAT results, whatever, the AVN, LV base or LV apex, pacing site. CV† was slowest in Scn5a+/– RVOT compared with the remaining Scn5a+/+ and WT, RV and RVOT recording sites. CV† in turn is determined by (4) the conduction velocity magnitude, CV* and (5) the dispersion in its direction CD, derived from local vector analysis. CV* was reduced with the Scn5a+/– as opposed to the WT genotype independently of the choice of RVOT as opposed to RV recording site with AVN pacing. In contrast, CD was increased by the choice of the RVOT as opposed to the RV as recording site, with no influence of or interaction with genotype, with AVN pacing. Further, biochemical and anatomical, studies demonstrated (6) a reduced Na,1.5 expression in both RVOT and RV of Scn5a+/– attributable solely to genotype and independent of anatomical region. In contrast, (7) interstitial fibrotic changes were greater in the RVOT than the RV in the Scn5a+/– but not WT, attributable to anatomical regions independent of genotype.

The results (1)–(7) together demonstrate and explain the highest incidences of arrhythmia in the Scn5a+/– RVOT. Admittedly, Scn5a+/– resulted in higher VERPs, nevertheless consistent with a reduced Na,1.5 expression, that would tend to increase AP wavelength, $\lambda$, contrary to expectations of arrhythmic substrate. Nevertheless, the observed differences in arrhythmogenicity, correlated with APs in the Scn5a+/– RVOT having the lowest CV† and CV*, and the highest CD amongst RV and RVOT recording sites in Scn5a+/– and WT hearts. Furthermore, the reductions in CV† reflect both the Scn5a+/– genotype, and the choice of RVOT as opposed to RV as a recording site. Of the component variables underlying CV†, reductions in CV* are attributable to the reduced Na,1.5 occurring in the Scn5a+/– genotype whereas the greater CD reflects the greater fibrosis in the RVOT compared with the RV.

These findings provide a physiological basis for clinical reports describing structural abnormalities and degenerative changes in patients with a SCNS5A mutation (Bezzina et al. 2003). For example, a boy with compound heterozygosity for two SCN5A mutations exhibited severe degenerative changes in the specialized conduction system (Bezzina et al. 2003). Fatty replacement and fibrosis in the RVOT have been described in patients with BrS despite normal LV anatomy (Thiene et al. 1988, Martini et al. 1989, Corrado et al. 1996). This often involved the RVOT free wall (Tada et al. 1998) at which VT or VF was most readily inducible during electrophysiological assessment (Carlson et al. 1994, Morita et al. 2003, Papavassiliu et al. 2004). They also extend recent reports of increased fibrosis with age particularly in male Scn5a+/– patients, although the latter studies did not distinguish between RV and RVOT fibrosis (Jeevaratnam et al. 2011, 2012).

Together, these results unify a number of clinical observations hitherto not fully studied using experimental models. They demonstrate a greater arrhythmic tendency in the RVOT of the Scn5a+/–, attributing this to a combination of reduced Na,1.5 expression and increased fibrosis. The increased arrhythmogenesis correlated with both (1) a reduced effective CV† associated with both reduced Na,1.5 expression and increased fibrosis in the RVOT. This in turn reflected (2) a reduced conduction velocity magnitude (CV*) associated only with reduced Na,1.5 expression and not increased fibrosis in the RVOT together with (3) an increased CD associated only with increased fibrosis in the RVOT and not reduced Na,1.5 expression. Both (2) and (3) combined in the case of the Scn5a+/– RVOT would thus increase its arrhythmic tendency in this analysis, that may be applicable to other arrhythmic conditions associated with both channel and structural abnormality (Duehmke et al. 2012, Heijman et al. 2013).

Conflict of interest

None declared.

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