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Differences in sino-atrial and atrio-ventricular function with age and sex attributable to the *Scn5a*$^{+/-}$ mutation in a murine cardiac model

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Abstract

Aim: To investigate the interacting effects of age and sex on electrocardiographic (ECG) features of *Scn5a*$^{+/-}$ mice modelling Brugada syndrome.

Methods: Recordings were performed on anaesthetized wild-type (WT) and *Scn5a*$^{+/-}$ mice and differences attributable to these risk factors statistically stratified.

Results: *Scn5a*$^{+/-}$ exerted sex-dependent effects upon sino-atrial function that only became apparent with age. RR intervals were greater in old male than in old female *Scn5a*$^{+/-}$. Atrio-ventricular (AV) conduction was slower in young female mice, whether WT and *Scn5a*$^{+/-}$, than the corresponding young male WT and *Scn5a*$^{+/-}$. However, PR intervals lengthened with age in male but not in female *Scn5a*$^{+/-}$ giving the greatest PR intervals in old male *Scn5a*$^{+/-}$ compared with either old male WT or young male *Scn5a*$^{+/-}$ mice. In contrast, PR intervals were similar in old female *Scn5a*$^{+/-}$ and in old female WT. QTc was prolonged in *Scn5a*$^{+/-}$ compared with WT, and female *Scn5a*$^{+/-}$ compared with female WT. Age-dependent alterations in durations of ventricular repolarization relative to WT affected male but not female *Scn5a*$^{+/-}$. Thus, T-wave durations were greater in old male *Scn5a*$^{+/-}$ compared with old male WT, but indistinguishable between old female *Scn5a*$^{+/-}$ and old female WT. Finally, analysis for combined interactions of genotype, age and sex demonstrated no effects on P wave and QRS durations and QTc intervals.

Conclusion: We demonstrate for the first time that age, sex and genotype exert both independent and interacting ECG effects. The latter suggest alterations in cardiac pacemaker function, atrio-ventricular conduction and ventricular repolarization greatest in ageing male *Scn5a*$^{+/-}$.

Keywords age, electrophysiology, sex, sodium channel, transgenic mice.

The Brugada (BrS) and Lev-Lenegre syndromes (progressive cardiac conduction defect, PCCD) (Kyndt *et al.* 2001) are both associated with altered cardiac conduction and increased cardiac arrhythmogenic risk potentially leading to sudden cardiac death. BrS is a leading cause of death of men under the age of 50 in endemic regions (Antzelevitch *et al.* 2002). Thus, men with BrS have a higher prevalence of life-threatening arrhythmias

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than women corresponding to incidences of 9.5% and 3.8% respectively (Sacher *et al.* 2008).

Brugada syndrome typically presents with a particular electrocardiographic (ECG) pattern of ST segment elevation in leads V1 to V3. However, female cases often show a less pronounced ST elevation or an absence of this spontaneous type 1 ECG pattern (Sacher *et al.* 2008). ECG patterns in BrS patients can fluctuate over time, altering between typical ST segment elevations or may even be transiently normal (Benito *et al.* 2008a). Conduction abnormalities may also occur in BrS as reflected in right bundle branch block, P wave and QRS duration widening, and PR interval prolongation observed in some patients (Brugada & Brugada 1992, Shimizu 2006). Recent reports have also described elongated PR intervals (Smits *et al.* 2002) and prolonged QRS durations (Takagi *et al.* 2007) in precordial ECG recordings. Furthermore, QRS durations were observed to be useful in stratifying BrS patients into high or low risk categories (Takagi *et al.* 2007). PCCD reflects a primary age-related degeneration in His-Purkinje conduction (Probst *et al.* 2003). It is associated with the ECG changes in right or left bundle branch block and widening QRS complexes in the absence of ST segment elevation. These changes progress to complete atrio-ventricular (AV) block causing syncope and sudden cardiac death (SCD) (Schott *et al.* 1999).

Both BrS and PCCD are associated with *SCN5A* haploinsufficiency. These mutations were identified in the *SCN5A* gene which encodes the α subunit of the cardiac Na^+ channel (locus 3p21, 28 exons) (Benito *et al.* 2008a). Males and females inherit this gene equally. However, only 18-30% of patients diagnosed with BrS are carriers of Na^+ channel mutations (Priori *et al.* 2000, Antzelevitch *et al.* 2005). Nevertheless 80% of patients in Western populations and 90% in Asian countries suffering from the ECG manifestations of BrS caused by *SCN5A* mutation are males (Shimizu 2004). Individuals with the *SCN5A* mutation exhibit P wave, PR and QRS durations prolongation that worsen with age (Probst *et al.* 2003). However, around 84% of carriers for *SCN5A* mutations have normal ECGs (Kyndt *et al.* 2001). Such incidences have not been established for PCCD.

The question as to which of the wide range of clinically observed age- and sex-related changes observed in BrS or Lev-Lenegre are the direct consequence of a modified *SCN5A* genotype is thus unclear. Nevertheless, a Na^+ channel genetic knockout involving *Scn5a*, whose features could be compared with otherwise genetically identical wild-type (WT), has recently been introduced to model these conditions. This heterozygous murine *Scn5a*$^{+/-}$ model shows prolonged P wave and PR durations, right axis deviation and second-degree AV block indicating conduction system deficiency (Papadatos *et al.* 2002). It has been reported that the duration of the QRS complex increased by 20% after 6 months from first measurement (Royer *et al.* 2005). The present study now proceeds to examine the implications of not only sex and age but also of their possible interactions for cardiac changes related to the *Scn5a*$^{+/-}$ mutation. Use of populations matched for both age and sex avoided the under-recruitment of female patients often occurring in *human* clinical trials studying SCD that may limit extrapolation of trial results to both sexes (Russo *et al.* 2009). It was then also possible to either implicate or discount a role for the *Scn5a*$^{+/-}$ mutation in the corresponding human ECG changes through their occurrence or otherwise in the animal system.

Methods

Mice were housed in an animal facility at 21 °C with 12 h light/dark cycles. Animals were fed sterile chow (RM3 Maintenance Diet; SDS, Witham, Essex, UK) and had free access to water. All procedures complied with the UK Home Office regulations [Animal (Scientific Procedures) Act 1986]. In order to develop a comprehensive risk assessment model of the two types of mice studied, a total of 22 WT and 20 *Scn5a*$^{+/-}$ knockout mice were used. All mice used for this study were derived from their respective 129/sv background strains to avoid any possible strain-related variation in the study. The mice were divided into eight groups. Group 1 was WT male aged 3 months (n = 5); Group 2 was WT male >12 months (n = 6); Group 3 was WT female 3 months (n = 5); Group 4 was WT female >12 months (n = 6); Group 5 was *Scn5a*$^{+/-}$ male aged 3 months (n = 5); Group 6 was *Scn5a*$^{+/-}$ male >12 months (n = 5); Group 7 was *Scn5a*$^{+/-}$ female 3 months (n = 5); and Group 8 was *Scn5a*$^{+/-}$ female >12 months (n = 5). A self-compounded sterilin was prepared using 1.8 mL ketamine hydrochloride at 100 mg mL^{-1} (Ketaset®; Fort Dodge, Southampton, UK) 0.35 mL of xylazine hydrochloride at 23.32 mg mL^{-1} (Rompun®; Bayer, Newbury, UK) and 2.85 mL of sterile phosphate base solution. The mice were then anaesthetized at a dose rate of 0.10 mL per 10 g body weight. Injection was given intraperitoneally with a 27G hypodermic needle into the left peritoneal cavity. The mice were tilted downwards (~30°) to ensure that visceral organs were avoided during drug injection. After injection the mice were placed in a dark cardboard cage and left undisturbed till the animal was under deep sedation.

For ECG recording, mice were placed in supine position on a heated platform to maintain a body temperature at 37 °C. Small strips of adhesive tapes

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were attached to the limbs to reduce any small movement thus reducing artefacts in ECG recordings. Two 2 mm needle electrodes (MLA1204; ADInstruments, Colorado Springs, CO, USA) were inserted in the right forelimb and the left hind limb respectively to produce Lead II recordings. The needle electrodes were connected to an input box in turn connected to an amplification (NL104) and filter (NL 126) unit mounted within a NL900D chassis and power supply (NeuroLog-Digitimer, Hertfordshire, UK). The low frequency cut-off point was set at 50 Hz and the high frequency cut-off point was set at 500 Hz. To further reduce frequency interference from the surroundings, the recording was performed in an iron chamber that had been grounded. Conversion of analog input to digital format was performed using the CED 1401 series (Cambridge Electronic Design, Cambridge, UK) connected to a computer. Spike II software (Cambridge Electronic Design) was used to record and subsequently analyse ECG recordings. ECG QT intervals obtained under these conditions were closely similar to previous reports of 90% action potential durations (APD_{90}) obtained from monophasic action potential readings in isolated perfused hearts (Stokoe *et al.* 2007). Upon completion of electrode placements, recordings were carried out for 15 min inclusive of 5 min for the stabilization of ECG recordings. Measurements for the respective ECG parameters were made from recordings obtained after the first 5 min only.

For all interval measurements, a standard criterion was used to ensure consistency. There is some variation between previous reports as to how such intervals are obtained from murine ECG traces. Figure 1a,b illustrates typical ECG recordings taken and the

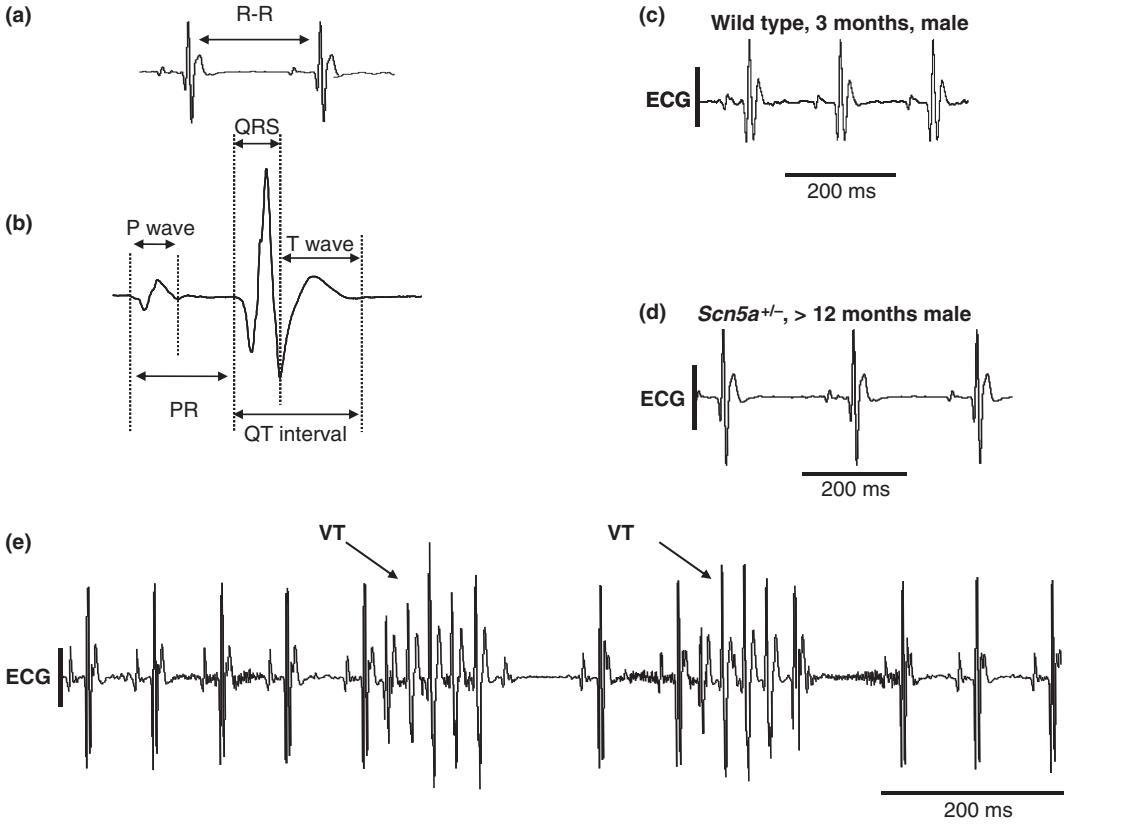

Figure 1 Lead II electrocardiographic traces obtained from anaesthetized wild-type (WT) and Scn5a$^{+/-}$ mice. Dipole recordings obtained with the negative electrode placed on the right fore limb and the positive electrode placed on the left hind limb. (a, b) The dotted lines indicate the intervals between (a) and within (b) successive electrocardiograms obtained using Spike II software (Cambridge Electronic Design). (c, d) Representative recordings from (c) young WT male (3 months) and (d) old *Scn5a*$^{+/-}$ male mice (>12 months). Mice were anaesthetized with ketamine and xylazine and placed on a heated platform during ECG recordings. (e) Occurrence of spontaneous non-sustained ventricular tachycardia (VT – indicated by arrow) was observed in one of the *Scn5a*$^{+/-}$ old female mouse. The VT lasted for approx. <5 s and did not degenerate into ventricular fibrillation. This mouse was >12 months old and appeared clinically healthy prior to commencement of experiment (date of birth: 28 March 2007; date of killing: 10 May 2008). It was housed in an animal facility at 21 °C with 12 h light/dark cycles. The mouse was fed sterile chow (RM3 Maintenance Diet; SDS) and had free access to water.

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intervals measured in the present study. Measurements were made by assigning specific cursors to the respective points on the ECG trace and intervals in milliseconds were generated by Spike II software (Cambridge Electronic Design). Analysis of ECG features in each group was made blinded and codes were only broken after the completion of Spike II analysis for the purpose of grouping and statistical analysis. The QTc interval was derived from the QT interval and this calculation was performed as described in a previously published report (Mitchell *et al.* 1998). The nonparametric statistical method using the Kruskal–Wallis test was chosen for this study. Statistical Package for Social Sciences v 16.0 [SPSS 16.0; SPSS (UK), Woking, Surrey, UK] was used in Kruskal–Wallis analysis, and STATA v10.1 (STATA Corporation, College Station, TX, USA) in multivariate linear regression model analysis of the results obtained from the recordings. $P < 0.05$ was considered to indicate significant difference and the data files were split for genotype, age and sex to further explore age and sex as interacting variables.

Results

Figure 1c,d compares typical ECG recordings from young (age 3 month) male WT (c) and old (>12 months) male *Scn5a*$^{+/-}$ (d) mice to represent the greatest phenotypic extremes encountered in the study. Recordings from all experimental groups showed similar waveforms with a regular sinus rhythm with no ECG abnormalities, permitting comparison of particular parameters between groups. The exception was one female *Scn5a*$^{+/-}$ old mouse that developed a spontaneous, non-sustained ventricular tachycardia (VT). Figure 1e illustrates this VT recording, which would be consistent with the expected phenotype from the *Scn5a*$^{+/-}$ mice modelling the BrS. Its relatively rare occurrence, in 1 of 20 *Scn5a*$^{+/-}$ mice, is consistent with the reported, rare occurrence of capturing spontaneous *Scn5a*$^{+/-}$-associated VT on human ECG recordings. Tables 1-4 summarize the results of the successive stratifications that permitted the following comparisons (1) by genotype (WT vs. *Scn5a*$^{+/-}$) alone (Table 1), (2) by genotype and age (3 months vs. >12 months) (Table 2), (3) by genotype and sex (male vs. female) (Table 3) and (4) following a full stratification by genotype, age and sex, that also permitted assessments of interactions between these parameters (Table 4). These yielded the following findings concerning each ECG parameter.

Figure 2a–f summarizes the results of a quantitative breakdown of the strategic intervals between successive features or durations of particular components of the observed ECG waveforms. It displays all these values obtained for each individual mouse in each population (total $n = 42$ animals) used for these experiments. The RR interval of one mouse was found to be scattered further away from the overall group (Fig. 2f) and when back-traced, this value was derived from the *Scn5a*$^{+/-}$ female mouse with the VT episode described above. Qualitative assessment through visual observation of the ECG recordings thus did not show any obvious further differences. However, major differences emerged with quantitative assessment.

Sino-atrial function

Of the four levels of stratification, differences in sino-atrial conduction as reflected in RR intervals were only observed in the final stratification by genotype, age and sex. Thus, sino-atrial function was compromised specifically in the old male *Scn5a*$^{+/-}$ relative to the old female *Scn5a*$^{+/-}$ mice (RR: 215.56 ± 17.03 ms vs. 155.70 ± 22.48 ms, $P = 0.047$). In contrast, they were indistinguishable between young male and young female WT, young male and young female *Scn5a*$^{+/-}$ and old male and old female WT.

Atrial conduction

Differences in atrial conduction were observed only in the stratification involving genotype and age. Thus, old *Scn5a*$^{+/-}$ mice showed significantly shorter P wave durations than young *Scn5a*$^{+/-}$ mice (16.08 ± 1.07 vs. 21.13 ± 1.35 ms, $P = 0.034$). In contrast, young *Scn5a*$^{+/-}$ mice had similar P wave durations as young WT mice. Ageing thus reduces a prolonged P wave duration initially observed in young *Scn5a*$^{+/-}$ to corresponding values similar to those observed in aged WT. Such differences were absent in all the other levels of stratification.

Table 1 Genotypic differences in wild-type (WT) and *Scn5a*$^{+/-}$ mice

Genotype	*n*	RR ms (±SE)	P wave ms (±SE)	PR ms (±SE)	QRS ms (±SE)	QTc ms (±SE)	T wave ms (±SE)
WT	22	210.4 (10.83)	20.24 (1.00)	41.87^{a} (1.20)	16.11 (0.44)	29.72^{b} (1.05)	26.1 (0.84)
Scn5a$^{+/-}$	20	183.4 (8.81)	18.61 (1.02)	46.67^{a} (1.07)	16.85 (0.57)	34.26^{b} (1.84)	28.53 (1.72)

Means with similar superscript differ significantly with each other at $P < 0.05$.

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Table 2 Electrocardiographic differences in wild-type (WT) and *Scn5a*$^{+/-}$ mice by age

		WT							*Scn5a*$^{+/-}$					
Genotype	*n*	RR ms (±SE)	P wave ms (±SE)	PR ms (±SE)	QRS ms (±SE)	QTc ms (±SE)	T wave ms (±SE)	*n*	RR ms (±SE)	P wave ms (±SE)	PR ms (±SE)	QRS ms (±SE)	QTc ms (±SE)	T wave ms (±SE)
Young	10	218.39 (19.50)	22.14 (0.97)	42.18 (1.69)	16.09 (0.84)	29.00 (2.10)	25.29 (1.28)	10	181.08 (7.08)	21.13^{a} (1.36)	44.58 (1.74)	16.78 (0.99)	31.16 (2.08)	25.13 (2.61)
Old	12	203.74 (11.93)	18.67 (1.52)	41.61$^{\lambda}$ (1.75)	16.13 (1.48)	30.32$^{\varphi}$ (0.87)	26.77$^{\omega}$ (1.12)	10	185.63 (16.62)	16.08^{a} (1.07)	48.75$^{\lambda}$ (0.94)	16.92 (0.64)	37.16$^{\varphi}$ (2.80)	31.93$^{\omega}$ (1.77)

Means with similar superscript differ significantly with each other at $P < 0.05$.

Table 3 Electrocardiographic differences in wild-type (WT) and *Scn5a*$^{+/-}$ mice by sex

		WT							*Scn5a*$^{+/-}$					
Genotype	*n*	RR ms (±SE)	P wave ms (±SE)	PR ms (±SE)	QRS ms (±SE)	QTc ms (±SE)	T wave ms (±SE)	*n*	RR ms (±SE)	P wave ms (±SE)	PR ms (±SE)	QRS ms (±SE)	QTc ms (±SE)	T wave ms (±SE)
Male	11	196.29 (9.06)	21.35 (1.28)	39.73$^{a,\omega}$ (1.42)	16.81$^{\pi}$ (0.54)	30.38 (1.27)	25.30 (1.14)	10	193.83 (11.83)	19.38 (1.77)	44.80$^{\omega}$ (1.55)	15.74$^{\pi}$ (0.36)	31.21 (1.77)	27.57 (2.67)
Female	11	224.51 (19.25)	19.14 (1.52)	44.02^{a} (1.76)	15.42 (0.64)	29.07$^{\lambda}$ (1.71)	26.90 (1.23)	10	172.87 (12.78)	17.83 (1.06)	48.53 (1.31)	17.96 (0.99)	37.32$^{\lambda}$ (3.01)	29.48 (2.24)

Means with similar superscript differ significantly with each other at $P < 0.05$.

Table 4 Electrocardiographic differences in wild-type (WT) and *Scn5a*$^{+/-}$ mice by both age and sex

			Male							Female					
Genotype	Age	*n*	RR ms (±SE)	P wave ms (±SE)	PR ms (±SE)	QRS ms (±SE)	QTc ms (±SE)	T wave ms (±SE)	*n*	RR ms (±SE)	P wave ms (±SE)	PR ms (±SE)	QRS ms (±SE)	QTc ms (±SE)	T wave ms (±SE)
WT	Young	5	184.99	22.50	38.11^{b}	17.12	31.36	24.97	5	251.79	21.78	46.26^{b}	15.06	26.65	25.62
			(15.42)	(1.05)	(1.22)	(0.78)	(2.21)	(2.09)		(30.25)	(1.80)	(1.75)	(1.43)	(3.53)	(1.70)
	Old	6	205.71	20.39	41.07$^{\varphi}$	16.54	29.56	25.57$^{\lambda}$	6	201.77	16.94	42.15	15.72	31.08	27.97
			(10.17)	(2.20)	(2.36)	(0.81)	(1.56)	(1.34)		(22.83)	(2.04)	(2.78)	(0.29)	(0.76)	(1.77)
Scn5a$^{+/-}$	Young	5	172.11	22.53	41.09^{a,c}	15.32	28.96	22.70	5	190.05	19.73	48.07^{c}	18.24	33.36	27.56
			(10.17)	(2.14)	(1.00)	(0.50)	(2.89)	(4.40)		(9.06)	(1.65)	(2.54)	(1.76)	(2.93)	(2.89)
	Old	5	215.56^{d}	16.22	48.52$^{a,\varphi}$	16.16	33.46	32.45$^{\lambda}$	5	155.70^{d}	15.94	48.98	17.68	41.27	31.41
			(17.03)	(2.14)	(1.68)	(0.49)	(1.78)	(1.23)		(22.48)	(0.74)	(1.05)	(1.14)	(4.95)	(3.52)

Means with similar superscript differ significantly with each other at $P < 0.05$.

Atrioventricular conduction

Stratification by genotype alone revealed that *Scn5a*$^{+/-}$ mice showed increased PR intervals compared with WT mice (PR: 41.87 ± 1.20 ms vs. 46.66 ± 1.07 ms $P = 0.007$), confirming previous reports (Papadatos *et al.* 2002). However, PR intervals in WT and *Scn5a*$^{+/-}$ mice were statistically indistinguishable in young animals. Nevertheless, PR intervals in the two genotypic groups diverged with age: they were greater in the old *Scn5a*$^{+/-}$ than in the old WT (PR: 48.75 ± 0.94 ms vs. 41.61 ± 1.75 ms, $P = 0.006$). Analysis by genotype and sex revealed opposing sex differences in the effect of the mutation on AV conduction. Thus, PR intervals were greater in the WT female than in the WT male population (PR: 44.02 ± 1.76 ms vs. 39.73 ± 1.42 ms, $P = 0.045$). PR intervals were also greater in the female *Scn5a*$^{+/-}$ than in the male *Scn5a*$^{+/-}$ but this result was not significant. The effect of the *Scn5a*$^{+/-}$ mutation on slowing AV conduction was greater in males than in females. Thus, on the one hand, PR intervals were greater in the *Scn5a*$^{+/-}$ males compared with WT males (PR: 44.80 ± 1.55 ms vs. 39.73 ± 1.42 ms, $P = 0.009$). On the other hand, although PR intervals were also greater in the female *Scn5a*$^{+/-}$ than the female WTs, this result was not significant.

The final stratification involving genotype, age and sex demonstrated that AV conduction was slower in young female mice compared with young males, regardless of genetic background. Thus, PR intervals were significantly greater in young female WT compared with young male WT mice (46.26 ± 1.75 ms vs. 38.11 ± 1.21 ms, $P = 0.009$). They were similarly greater in young female *Scn5a*$^{+/-}$ than young male *Scn5a*$^{+/-}$ mice (48.07 ± 2.54 ms vs. 41.09 ± 1.01 ms, $P = 0.047$).

It was additionally observed that AV conduction in young male carriers of the *Scn5a*$^{+/-}$ mutation was normal but slowed with age. Thus, ageing males carrying the *Scn5a*$^{+/-}$ mutation showed increases in PR intervals from 41.09 ± 1.00 to 48.52 ± 1.68 ms ($P = 0.009$). In contrast, the male WT mice showed no such detectable changes in PR intervals with age. Furthermore, PR intervals were similar in young male WT and young male *Scn5a*$^{+/-}$ mice. Together these findings were consistent with the final observation that old male *Scn5a*$^{+/-}$ showed significantly longer PR intervals compared with old male WT (41.07 ± 2.36 ms) ($P = 0.028$). Female mice showed a markedly contrasting pattern in PR interval with age. Thus, regardless of genotype, they did not show deteriorations in PR intervals with age in contrast to the findings in males. Thus, there were no significant differences in PR intervals for either old female WT or old female

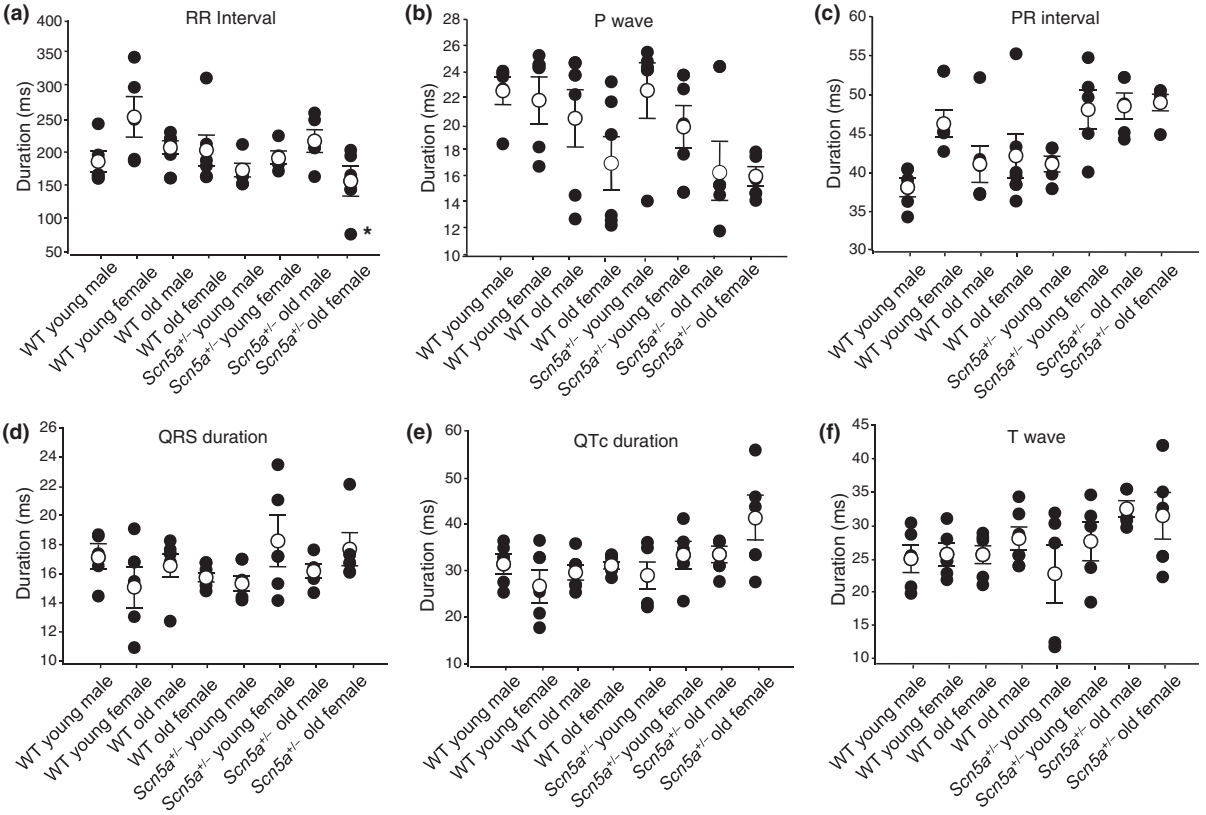

Figure 2 Individual electrocardiographic data sets for WT (n = 22) and *Scn5a*$^{+/-}$ (n = 20) mice used for ECG recordings. Complete description of the data acquired for every mouse studied. A descriptive view of mean distributions for (a) RR interval, (b) P wave duration, (c) PR interval, (d) QRS duration, (e) QTc duration and (f) T wave duration demonstrated that all mice had mean values that were similar to their respective groupings. One *Scn5a*$^{+/-}$ mouse was observed to have an abnormally short R-R duration in comparison with the rest of the study group and when traced back, corresponded to the mouse with the VT described in Figure 1e.

Scn5a$^{+/-}$ when compared with the respective old male WT or *Scn5a*$^{+/-}$.

Ventricular depolarization

QRS durations were longer in the WT male when these were compared with the *Scn5a*$^{+/-}$ male (QRS: 16.81 ± 0.54 ms vs. 15.74 ± 0.36 ms, P = 0.041). In contrast, there were no significant differences in QRS durations between sexes in either the WT or the *Scn5a*$^{+/-}$ populations. The other stratification analysis by just genotype, genotype and age, and genotype, age and sex all revealed no differences in ventricular depolarization.

Combined ventricular depolarization and repolarization phase

Stratification by genotype alone revealed greater QTc intervals in *Scn5a*$^{+/-}$ compared with WT mice (QTc: 29.72 ± 1.05 ms vs. 34.26 ± 1.84 ms, P = 0.047). Stratification by genotype and age revealed that QTc intervals in WT and *Scn5a*$^{+/-}$ mice were statistically indistinguishable in young animals. However, the two genotypic groups diverged with age. Thus, we observed greater QTc intervals in the older *Scn5a*$^{+/-}$ than in the older WT (QTc: 37.16 ± 2.80 ms vs. 30.32 ± 0.87 ms, P = 0.021). When stratified by genotype and sex, the QTc intervals were greater in *Scn5a*$^{+/-}$ compared with WT females (QTc: 37.32 ± 3.01 ms vs. 29.07 ± 3.01 ms, P = 0.035). However, there were no differences in QTc intervals between groups stratified by their genotype, age and sex.

Ventricular repolarization

The initial stratification analysis by just genotype and then by genotype and sex revealed no differences in ventricular depolarization. Thus, T wave durations in WT and *Scn5a*$^{+/-}$ mice were statistically indistinguishable in young animals. However, stratification by genotype and age revealed T wave durations in the two genotypic groups that diverged with age. Thus, they were greater in the old *Scn5a*$^{+/-}$ than in the old WT (T wave: 31.93 ± 1.77 ms vs. 26.77 ± 1.12 ms, P = 0.021).

We further observed that, in the final stratification, by genotype, age and sex, durations of ventricular repolarization were greater in old male *Scn5a*$^{+/-}$ compared with old male WT, but similar in old female *Scn5a*$^{+/-}$ and old female WT. Thus, T-wave durations for the *Scn5a*$^{+/-}$ old male were significantly greater than the corresponding value found in the WT old male (32.45 ± 1.23 ms vs. 25.57 ± 1.34 ms, $P = 0.006$).

Discussion

The present experiments investigated the influence of both age and sex differences on electrophysiological phenotypes in systematically grouped intact heterozygous *Scn5a*$^{+/-}$ and WT mice for the first time. They extend previous studies (Papadatos *et al.* 2002, Royer *et al.* 2005) and demonstrate the major effects of both age and sex as contributing or interacting factors in the physiological differences between *Scn5a*$^{+/-}$ and WT for the first time. This was accomplished by studying larger *Scn5a*$^{+/-}$ and WT populations that could be segmented by sex and age, which examined a wider range of ECG parameters. This made it additionally possible to either implicate or discount roles for the *Scn5a*$^{+/-}$ mutation in the corresponding clinical ECG changes (see introduction) through their occurrence or otherwise in the animal system.

In our analysis of the results from the recordings, the nonparametric statistical method using the Kruskal–Wallis test was used in this study. $P < 0.05$ was considered to indicate significant difference and the data files were split for genotype, age and sex to further explore age and sex as interacting variables. Our use of such a robust nonparametric method permitted comparison of groups each containing sample sizes of < 30 per group, avoiding assumptions of normal distributions. Each successive stratification represents a new grouping of mice being examined and thus corresponds to a different research question at every level examined. Therefore, even if an association is not found at one level, this does not negate an association being found at a different level. Our results for ECG parameters suggest that age and sex act as interacting variables, e.g. the association between ECG parameter and genotype varies at different age levels (old vs. young) and between the sexes (males vs. females). For example, if we examine the QTc data, we find a significant association between QTc and the two genotypes (Table 1). However, if we then stratify the data by age, the association remains significant only for the old mice (Table 2). Similarly, if we stratify the data by sex, the association remains significant in females but not in males (Table 3). In Table 4, the final stratification allows us to make a number of additional comparisons in groups broken down by both age and sex, again demonstrating the interactive effect of these variables.

We nevertheless conducted a series of multivariate linear regression models to examine the association between each ECG parameter and genotype, adjusting for age and sex. The association between each ECG parameter and genotype remained the same after adjustment for age and sex, suggesting that these variables are not *confounders* of the relationship between ECG parameters and genotype. Furthermore, running a multivariate model with age and sex as covariates does not allow us to tease out the independent *interacting* effects of age and sex, hence we have presented Kruskal–Wallis group comparisons separately by age and sex to explore this trend. We therefore did not adopt this multivariate statistical analysis model in our study.

First, sino-atrial pacemaker function was compromised, leading to increased RR intervals in older male compared with older female *Scn5a*$^{+/-}$. Thus, although age or sex did not affect RR interval when considered independently, when considered together these resulted in a bradycardic phenotype in old male *Scn5a*$^{+/-}$. The present results thus provide a physiological basis implicating Na^+ channel mutations for recent clinical reports associating bradycardic episodes in both asymptomatic and symptomatic BrS patients with augmented ST segment elevation (Mizumaki *et al.* 2006). Both phenomena occur more often in older male patients (Brugada *et al.* 2002, 2003, Eckardt *et al.* 2005).

Secondly, intra-atrial conduction, reflected in P wave durations, was prolonged in young *Scn5a*$^{+/-}$ compared with old *Scn5a*$^{+/-}$ mice. Such a finding may have been masked in the earlier studies that may have grouped mutants together regardless of age and sex (Papadatos *et al.* 2002, Royer *et al.* 2005).

Thirdly, AV conduction, reflected in PR intervals, was slower in *both* the young female WT and the young female *Scn5a*$^{+/-}$ relative to the respective male animals. As this PR interval prolongation applied to *both* WT and *Scn5a*$^{+/-}$, it could therefore be attributed to actions of the female hormone, oestrogen, rather than the presence or absence of a *Scn5a*$^{+/-}$ genotype, on cardiac conduction. Our study compared young mice at the early to peak period of their reproductive cycle with older, >12 month, mice that had reached the end of their reproductive cycle, which would be expected to be accompanied by a decline in oestrogen levels (Silver 1995). Saba *et al.* (2002) reported that oestrogen replacement therapy prolonged PR intervals and shortened right ventricular refractory periods in ovariectomized mice (Saba *et al.* 2002). These differences would contrast with the relatively sustained levels of testosterone in male mice through similar ages.

Fourthly, AV conduction was also slower in *Scn5a*$^{+/-}$ compared with WT mice when these were considered as

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a whole. Further stratifications of experimental groups additionally demonstrated that PR intervals were longer in the old *Scn5a*$^{+/-}$ compared with their WT counterparts. They finally identified this difference in PR intervals with the old male *Scn5a*$^{+/-}$ mice. These findings contrast with the observations that intra-atrial conduction, reflected in P wave durations in such old male *Scn5a*$^{+/-}$, was not significantly different from those observed in the young male *Scn5a*$^{+/-}$. Nevertheless, taken together these findings attribute the increased PR durations specifically to disruptions in the AV node. Thus, old male *Scn5a*$^{+/-}$ mice show a greater vulnerability to AV node conduction disorder in comparison with old female *Scn5a*$^{+/-}$ and all the WT. This pattern of PR changes with age, sex and/or genotype, in our experimental system, provide an electrophysiological basis, attributable to the *Scn5a*$^{+/-}$ mutation, for corresponding changes previously observed in human BrS. These latter studies attributed the stronger BrS phenotype in males to more prominent right ventricular epicardial transient outward currents, I_{to}, brought about by the hormonal action of testosterone (Di Diego *et al.* 2002). Conversely, orchiectomy abolished ST elevation in BrS patients (Matsuo *et al.* 2003). Others reported an absence of correlations between ST segment elevation and testosterone but higher levels of this hormone in BrS compared with normal males (Shimizu *et al.* 2007). It has also been reported that testosterone administration increased net outward currents thus increasing the occurrence of the BrS phenotype (Shuba *et al.* 2001, Liu *et al.* 2003).

Fifthly, durations of ventricular repolarization in our initial stratification that were confined to the effect of sex showed that T wave durations were prolonged in the old *Scn5a*$^{+/-}$ compared with the old WT. The subsequent stratification isolated this prolongation specifically to the old male Scn5a$^{+/-}$ mice where it was observed that T wave durations were greater in old male *Scn5a*$^{+/-}$ compared with old male WT, but were similar in aged female *Scn5a*$^{+/-}$ when compared with old female WT. Prolonged ventricular repolarization is a known risk factor for ischaemic heart disease and cardiac-related mortality. This finding thus suggests that older males carrying the *SCN5A*$^{+/-}$ mutation are potentially at higher clinical risk for the aforementioned condition compared with older males who are non-carriers. More importantly this finding provides evidence that the *Scn5a* mutation may not only cause SUDS but could also lead to various other cardiac-related mortalities.

Finally, QTc durations were increased in the old *Scn5a*$^{+/-}$ group compared with the old WT group in our initial stratification. When examined specifically for the influence of sex, the *Scn5a*$^{+/-}$ females showed a QTc prolongation compared with the corresponding WT females. In contrast, the *Scn5a*$^{+/-}$ males showed QTc intervals that were similar to the corresponding WT males. This preferential prolongation of QTc durations in the mutant females parallels clinical findings that sodium channel blockers significantly increased QTc intervals in female BrS patients (Benito *et al.* 2008b). A human clinical study observed QTc prolongation in ECG of patients administered flecainide (Pitzalis *et al.* 2003). In view of the known inhibitory effect of flecainide on Na^+ channels, this would suggest that such a QTc prolongation reflects downregulatory changes in Na^+ channel expression. However, full stratifications by sex and age yielded no differences in QTc durations amongst the resulting groups. Thus, our carefully designed study made it possible to prove that age and sex are independent factors associated with QTc prolongation in the *Scn5a*$^{+/-}$ mice.

In conclusion, our investigation separated both independent and interacting effects of age, sex and the *Scn5a*$^{+/-}$ genotype on ECG features of anaesthetized 129sv mice. Taken together our findings demonstrate for the first time that age, sex and genotype each exert varying effects on the observed ECG parameters. When combined, they thus result in altered cardiac pacemaker function, AV conduction and ventricular repolarization in ageing male *Scn5a*$^{+/-}$ in parallel with clinical findings. These latter effects thus extend simple biophysical predictions of uniform reductions in cardiac conduction expected from the loss of Na^+ channel function.

Conflict of interest

The authors have no conflicts of interest.

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References

Antzelevitch, C., Brugada, P., Brugada, J., Brugada, R., Shimizu, W., Gussak, I. & Perez Riera, A.R. 2002. Brugada syndrome: a decade of progress. *Circ Res* **91**, 1114–1118.

Antzelevitch, C., Brugada, P., Borggrefe, M., Brugada, J., Brugada, R., Corrado, D., Gussak, I., LeMarec, H., Nademanee, K., Perez Riera, A.R., Shimizu, W., Schulze-Bahr, E., Tan, H. & Wilde, A. 2005. Brugada syndrome: report of the second consensus conference: endorsed by the Heart Rhythm Society and the European Heart Rhythm Association. *Circulation* **111**, 659–670.

Benito, B., Brugada, R., Brugada, J. & Brugada, P. 2008a. Brugada syndrome. *Prog Cardiovasc Dis* **51**, 1–22.

 doi: 10.1111/j.1748-1716.2010.02110.x

Benito, B., Sarkozy, A., Mont, L., Henkens, S., Berruezo, A., Tamborero, D., Arzamendi, D., Berne, P., Brugada, R., Brugada, P. & Brugada, J. 2008b. Gender differences in clinical manifestations of Brugada syndrome. *J Am Coll Cardiol* **52**, 1567–1573.

Brugada, P. & Brugada, J. 1992. Right bundle branch block, persistent ST segment elevation and sudden cardiac death: a distinct clinical and electrocardiographic syndrome. A multicenter report. *J Am Coll Cardiol* **20**, 1391–1396.

Brugada, J., Brugada, R., Antzelevitch, C., Towbin, J., Nademanee, K. & Brugada, P. 2002. Long-term follow-up of individuals with the electrocardiographic pattern of right bundle-branch block and ST-segment elevation in precordial leads V1 to V3. *Circulation* **105**, 73–78.

Brugada, J., Brugada, R. & Brugada, P. 2003. Determinants of sudden cardiac death in individuals with the electrocardiographic pattern of Brugada syndrome and no previous cardiac arrest. *Circulation* **108**, 3092–3096.

Di Diego, J.M., Cordeiro, J.M., Goodrow, R.J., Fish, J.M., Zygmunt, A.C., Perez, G.J., Scornik, F.S. & Antzelevitch, C. 2002. Ionic and cellular basis for the predominance of the Brugada syndrome phenotype in males. *Circulation* **106**, 2004–2011.

Eckardt, L., Probst, V., Smits, J.P., Bahr, E.S., Wolpert, C., Schimpf, R., Wichter, T., Boisseau, P., Heinecke, A., Breithardt, G., Borggrefe, M., LeMarec, H., Bocker, D. & Wilde, A.A. 2005. Long-term prognosis of individuals with right precordial ST-segment-elevation Brugada syndrome. *Circulation* **111**, 257–263.

Kyndt, F., Probst, V., Potet, F., Demolombe, S., Chevallier, J.C., Baro, I., Moisan, J.P., Boisseau, P., Schott, J.J., Escande, D. & Le Marec, H. 2001. Novel SCN5A mutation leading either to isolated cardiac conduction defect or Brugada syndrome in a large French family. *Circulation* **104**, 3081–3086.

Liu, X.K., Katchman, A., Whitfield, B.H., Wan, G., Janowski, E.M., Woosley, R.L. & Ebert, S.N. 2003. In vivo androgen treatment shortens the QT interval and increases the densities of inward and delayed rectifier potassium currents in orchiectomized male rabbits. *Cardiovasc Res* **57**, 28–36.

Matsuo, K., Akahoshi, M., Seto, S. & Yano, K. 2003. Disappearance of the Brugada-type electrocardiogram after surgical castration: a role for testosterone and an explanation for the male preponderance. *Pacing Clin Electrophysiol* **26**, 1551–1553.

Mitchell, G.F., Jeron, A. & Koren, G. 1998. Measurement of heart rate and Q-T interval in the conscious mouse. *Am J Physiol* **274**, H747–H751.

Mizumaki, K., Fujiki, A., Nishida, K., Sakabe, M., Tsuneda, T., Sugao, M., Iwamoto, J., Nagasawa, H. & Inoue, H. 2006. Bradycardia-dependent ECG changes in Brugada syndrome. *Circ J* **70**, 896–901.

Papadatos, G.A., Wallerstein, P.M., Head, C.E., Ratcliff, R., Brady, P.A., Benndorf, K., Saumarez, R.C., Trezise, A.E., Huang, C.L., Vandenberg, J.I., Colledge, W.H. & Grace, A.A. 2002. Slowed conduction and ventricular tachycardia after targeted disruption of the cardiac sodium channel gene Scn5a. *Proc Natl Acad Sci USA* **99**, 6210–6215.

Pitzalis, M.V., Anaclerio, M., Iacoviello, M., Forleo, C., Guida, P., Troccoli, R., Massari, F., Mastropasqua, F., Sorrentino, S., Manghisi, A. & Rizzon, P. 2003. QT-interval prolongation in right precordial leads: an additional electrocardiographic hallmark of Brugada syndrome. *J Am Coll Cardiol* **42**, 1632–1637.

Priori, S.G., Napolitano, C., Gasparini, M., Pappone, C., Della Bella, P., Brignole, M., Giordano, U., Giovannini, T., Menozzi, C., Bloise, R., Crotti, L., Terreni, L. & Schwartz, P.J. 2000. Clinical and genetic heterogeneity of right bundle branch block and ST-segment elevation syndrome: a prospective evaluation of 52 families. *Circulation* **102**, 2509–2515.

Probst, V., Kyndt, F., Potet, F., Trochu, J.N., Mialet, G., Demolombe, S., Schott, J.J., Baro, I., Escande, D. & Le Marec, H. 2003. Haploinsufficiency in combination with aging causes SCN5A-linked hereditary Lenegre disease. *J Am Coll Cardiol* **41**, 643–652.

Royer, A., van Veen, T.A., Le Bouter, S., Marionneau, C., Griol-Charhbili, V., Leoni, A.L., Steenman, M., van Rijen, H.V., Demolombe, S., Goddard, C.A. *et al.* 2005. Mouse model of SCN5A-linked hereditary Lenegre's disease: age-related conduction slowing and myocardial fibrosis. *Circulation* **111**, 1738–1746.

Russo, A.M., Day, J.D., Stolen, K., Mullin, C.M., Doraiswamy, V., Lerew, D.L. & Olshansky, B. 2009. Implantable cardioverter defibrillators: do women fare worse than men? Gender comparison in the INTRINSIC RV trial. *J Cardiovasc Electrophysiol* **20**, 973–978.

Saba, S., Zhu, W., Aronovitz, M.J., Estes, N.A., 3rd, Wang, P.J., Mendelsohn, M.E. & Karas, R.H. 2002. Effects of estrogen on cardiac electrophysiology in female mice. *J Cardiovasc Electrophysiol* **13**, 276–280.

Sacher, F., Meregalli, P., Veltmann, C., Field, M.E., Solnon, A., Bru, P., Abbey, S., Jais, P., Tan, H.L., Wolpert, C. *et al.* 2008. Are women with severely symptomatic Brugada syndrome different from men? *J Cardiovasc Electrophysiol* **19**, 1181–1185.

Schott, J.J., Alshinawi, C., Kyndt, F., Probst, V., Hoorntje, T.M., Hulsbeek, M., Wilde, A.A., Escande, D., Mannens, M.M. & Le Marec, H. 1999. Cardiac conduction defects associate with mutations in SCN5A. *Nat Genet* **23**, 20–21.

Shimizu, W. 2004. Gender difference and drug challenge in Brugada syndrome. *J Cardiovasc Electrophysiol* **15**, 70–71.

Shimizu, W. 2006. Does an overlap syndrome really exist between Brugada syndrome and progressive cardiac conduction defect (Lenegre syndrome)? *J Cardiovasc Electrophysiol* **17**, 276–278.

Shimizu, W., Matsuo, K., Kokubo, Y., Satomi, K., Kurita, T., Noda, T., Nagaya, N., Suyama, K., Aihara, N., Kamakura, S., Inamoto, N., Akahoshi, M. & Tomoike, H. 2007. Sex hormone and gender difference – role of testosterone on male predominance in Brugada syndrome. *J Cardiovasc Electrophysiol*, **18**, 415–421.

Shuba, Y.M., Degtiar, V.E., Osipenko, V.N., Naidenov, V.G. & Woosley, R.L. 2001. Testosterone-mediated modulation of HERG blockade by proarrhythmic agents. *Biochem Pharmacol*, **62**, 41–49.

Silver, L.M. 1995. *Mouse Genetics: Concepts and Applications*. Oxford University Press, New York.

Journal compilation © 2010 Scandinavian Physiological Society, doi: 10.1111/j.1748-1716.2010.02110.x

Smits, J.P., Eckardt, L., Probst, V., Bezzina, C.R., Schott, J.J., Remme, C.A., Haverkamp, W., Breithardt, G., Escande, D., Schulze-Bahr, E., LeMarec, H. & Wilde, A.A. 2002. Genotype-phenotype relationship in Brugada syndrome: electrocardiographic features differentiate SCN5A-related patients from non-SCN5A-related patients. *J Am Coll Cardiol* **40**, 350–356.

Stokoe, K.S., Balasubramaniam, R., Goddard, C.A., Colledge, W.H., Grace, A.A. & Huang, C.L. 2007. Effects of flecainide and quinidine on arrhythmogenic properties of Scn5a$^{+/-}$ murine hearts modelling the Brugada syndrome. *J Physiol* **581**, 255–275.

Takagi, M., Yokoyama, Y., Aonuma, K., Aihara, N. & Hiraoka, M. 2007. Clinical characteristics and risk stratification in symptomatic and asymptomatic patients with brugada syndrome: multicenter study in Japan. *J Cardiovasc Electrophysiol* **18**, 1244–1251.

