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Role of common and rare variants in *SCN10A*: results from the Brugada syndrome QRS locus gene discovery collaborative study

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Aims Brugada syndrome (BrS) remains genetically heterogeneous and is associated with slowed cardiac conduction. We aimed to identify genetic variation in BrS cases at loci associated with QRS duration.

Methods and results A multi-centre study sequenced seven candidate genes (*SCN10A*, *HAND1*, *PLN*, *CASQ2*, *TKT*, *TBX3*, and *TBX5*) in 156 Caucasian *SCN5A* mutation-negative BrS patients (80% male; mean age 48) with symptoms (64%) and/or a family history of sudden death (47%) or BrS (18%). Forty-nine variants were identified: 18 were rare (MAF $<1\%$) and non-synonymous; and 11/18 (61.1%), mostly in *SCN10A*, were predicted as pathogenic using multiple bioinformatics tools. Allele frequencies were compared with the Exome Sequencing and UK10K Projects. SKAT methods tested rare variation in *SCN10A* finding no statistically significant difference between cases and controls. Co-segregation analysis was possible for four of seven probands carrying a novel pathogenic variant. Only one pedigree (I671V/G1299A in *SCN10A*) showed co-segregation. The *SCN10A* SNP V1073 was, however, associated strongly with BrS [66.9 vs. 40.1% (UK10K) OR (95% CI) = 3.02 (2.35−3.87), $P = 8.07 \times 10-19$]. Voltage-clamp experiments for $Na_V1.8$ were performed for *SCN10A* common variants V1073, A1073, and rare variants of interest: A200V and I671V. V1073, A200V and I671V, demonstrated significant reductions in peak I_{Na} compared with ancestral allele A1073 (rs6795970).

Conclusion Rare variants in the screened QRS-associated genes (including *SCN10A*) are not responsible for a significant proportion of *SCN5A* mutation negative BrS. The common SNP *SCN10A* V1073 was strongly associated with BrS and demonstrated loss of $Na_V1.8$ function, as did rare variants in isolated patients.

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1. Introduction

Brugada syndrome (BrS) is a primary arrhythmia syndrome with an increased risk of sudden cardiac death (SCD). The diagnostic ECG is the type 1 pattern seen in at least one of the right precordial ECG leads.[1] This may present spontaneously or may require fever or a sodium channel blocker to provoke it. It is a genetic condition with male predominance.[1] Loss-of-function mutations in *SCN5A*, encoding the canonical cardiac sodium channel, are found in ~20% of index cases.[2] Other genes harbouring potentially causative mutations have been described but only infrequently.[2] Thus, in the majority of BrS the genetic aetiology is unexplained.

The BrS phenotype is often associated with intraventricular conduction delay. A recent genome-wide association study (GWAS) comparing subjects with BrS to population controls highlighted common variants at the QRS duration associated loci rs10428132 (*SCN10A*) and rs11708996 (*SCN5A*),[4] as risk alleles. These data support the hypothesis that loci associated with QRS duration in the general population[3] may harbour genetic variation that contributes to inheritance of BrS. Thus, this multi-centre collaboration aimed to determine the prevalence, association, and functional consequences of coding variants in *SCN10A* and biologically plausible candidate genes in 165 well-characterized *SCN5A* mutation-negative BrS index cases enriched for symptomatic and familial risk.

2. Methods

2.1 Case cohort

Unrelated index cases of BrS were provided by eight clinical centres: 34 Paris, 30 Pavia, 27 Amsterdam, 24 Nantes, 22 London, 17 Copenhagen, 6 Munich, and 5 Nashville. Inclusion criteria were:

- Self-reported European ancestry;
- *SCN5A* mutation negative;
- Type 1 pattern in at least one of the standard and/or high right precordial ECG leads with or without sodium channel blocker challenge.

At least one of the following was also required:

- Prior cardiac arrest;
- Documented spontaneous polymorphic ventricular tachycardia or fibrillation;
- Cardiac syncope (non-vagal and tilt test negative if undertaken);
- A family history of premature adult SCD <40 years;
- A family history of autopsy negative unexplained SCD;
- Other relatives with type 1 pattern.

Cases were excluded if any of the following were present:

- Significant structural cardiac disease;
- Drug-associated presentations without reproduction by a sodium channel blocker challenge;
- Isolated type 1 pattern;
- A family history of sudden infant death syndrome only.

Of the 165 cases included initially, two were excluded after *SCN5A* re-sequencing revealed a mutation, four due to revision of the diagnosis, and three because of non-European ancestry. The final sample size was 156 cases (*Table 1*). Other than 12 cases from London, the rest had been included in the BrS GWAS.[4] All ECGs were digitized and analysed using ImageJ (http://imagej.nih.gov/ij/). Up to three consecutive sinus beats in lead II (or V5) were used to measure PR, QRS, QT, and preceding RR, and averaged.

2.2 Gene selection and genotyping

Seven genes (*SCN10A*, *HAND1*, *CASQ2*, *TKT*, *PLN*, *TBX5*, *TBX3*) associated with the QRS duration in a recent large scale meta-analysis of GWAS[3] were selected based upon the strength of the GWAS signal, biological plausibility of a functional effect in BrS and/or a prior description of monogenic disease (see Supplementary material online, *Methods* for further details).

Genomic DNA was extracted at each participating site. Samples underwent comprehensive mutational analysis of all amino acid coding exons and exon/intron boundaries using polymerase chain reaction and Sanger sequencing (Supplementary material online, *Table S1*: PCR primers).

2.3 Determination of predicted pathogenicity for novel mutations

Putative pathogenicity was assigned based on the following criteria: (i) a non-synonymous (NS) variant; (ii) absent in at least 4300 European ancestry

Table 1 Clinical characteristics of BrS index cases and UK10K control samples included in the case–control analysis

Clinical characteristics of index cases included in the analysis	Value (± SD) BrS cases	Value (± SD) UK10K
Age, years[a]	48 (± 14)	55 (± 11)
Male (%)	125 (80)	0
Spontaneous type 1 ECG pattern[b] (%)	72 (46)	NA
Type 1 ECG pattern induced by intravenous sodium channel blocker[b] (%)	84 (54)	NA
PR interval[c], ms	173 (± 27)	159 (± 23)
QRS interval[c], ms	101 (± 17)	88 (± 10)
RR interval, ms	845 (± 159)	915 (± 146)
QTc interval (Bazett's correction), ms	406 (± 32)	427 (± 21)
Symptomatic (resuscitated cardiac arrest and/or syncope) (%)	100 (64)	–
Resuscitated cardiac arrest (%)	16 (10)	–
Syncope with documented VT or VF (%)	18 (12)	–
Syncope without documented VT or VF (%)	68 (44)	–
Family history of sudden death (%)	74 (47)	–
Familiy history of type 1 ECG pattern (%)	28 (18)	–

[a]At diagnosis for BrS cases.
[b]In at least one right-ventricular ECG lead in 2nd, 3rd, and 4th intercostal spaces.
[c]Significant difference for these intervals between cases and controls ($P < 0.001$).

controls (8600 reference alleles) from the National Heart, Lung, and Blood Institute (NHLBI) Grand Opportunity (GO) Exome Sequencing Project (ESP) (https://esp.gs.washington.edu/drupal/); and (iii) predicted pathogenic by at least three of the *in-silico* prediction tools as described by Giudicessi *et al.* [Grantham score,[5] GERP,[6] PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/), and SIFT (http://sift.jcvi.org/www/SIFT_chr_coords_submit.html)].[7]

2.4 Pedigrees investigated

When a putative pathogenic variant was identified in a proband, their living relatives were offered mutation analysis for the same variant. Segregation of phenotype and genotype was then analysed.

2.5 Analysis of rare variants using controls from the NHLBI GO ESP

The ESP control group consisted of 4300 individuals of European American ancestry. Genotype data from this cohort were used in a case–control analysis for all variants identified during this project using Pearson's χ^2 test. Results were deemed significant if *P*-values were below a Bonferroni-corrected α level of 0.001(0.05/50), based on the number of variants tested for.

2.6 Analysis of common variants using UK10K control samples (http://www.uk10k.org)

To formally test the association of variants with BrS, we performed a case–control association analysis using all BrS cases and a control population drawn from the TwinsUK study,[8] a subset of the UK10K cohort (UK10K, 2010) samples. The control population ($n = 1279$) included only healthy individuals of European ancestry in whom the type 1 BrS ECG pattern was absent (*Table 1*). Details of genotyping methods and sample quality control have been presented elsewhere.[9] Samples ECGs were available and were measured as previously reported.[3]

2.7 Association testing

Genotypes for all variants >1% frequency identified in the BrS cases were matched with genotypes in TwinsUK. All variants were tested using logistic regression and assuming an additive genetic model in SNPtest (version 2.4.1), without additional covariates,[4,10] and checked for quality (info score >0.95, HWE *P*-value $>1 \times 10^{-6}$, call rate >95%).

2.8 Rare-variant association testing

We ran two collapsed rare-variant tests for all variants in *SCN10A*: the sequence kernel association test (SKAT)[11] and an optimized version of this test (SKAT-O),[12] which behaves automatically as a burden test or as a sequence kernel association depending on which is more powerful. Each test was run either with default weights, in which variants with a lower frequency are assigned a higher weight according to the beta distribution (dbeta function in R), or with frequency and functional weights[13] (Supplementary material online, *Table S2*), defined as the product of the allele frequency weight and the scaled CADD score. The underlying idea is that for the latter, we up-weight rare variants, but also assign more weight to those rare variants predicted to have a functional effect, potentially increasing the power of these tests. We also collapsed uncommon [1–5%] and rare variants [<1%] separately.

Bonferroni corrections were used to define statistical significance levels. We tested only the *SCN10A* gene, one phenotype, and three frequency strata (all, uncommon, and rare variants), using either default and combined frequency and functional weights, and two different statistical tests. Therefore, tests were deemed significant if *P*-values were below a value of $\alpha = 0.05/3$ frequency strata/two weighing methods/two different statistical tests $= 4.2 \times 10^{-3}$.

2.9 Functional expression: *SCN10A* variants

As described subsequently, our analysis implicated mainly *SCN10A* variation in BrS, a high-priority gene arising from the QRS and BrS GWAS. Therefore, for cases with a potential pathogenic variant in *SCN10A* and/or evidence for segregation, we undertook functional expression using site-directed mutagenesis and *in-vitro* electrophysiological voltage-clamp studies to establish whether these variants perturb the function of the $Na_v1.8$ current encoded by *SCN10A* (see Supplementary material online, *Methods*). The NS common variant most significantly associated with BrS in the case–control study (see in what follows) was also studied.

ND7/23 cells were transfected transiently with plasmids containing *SCN10A* carrying these variants. Experiments were conducted at room temperature after 48 h incubation at 37°C. All recordings were performed in the presence of tetrodotoxin (TTX) 200 nM, nisoldipine 1 μM, and NiCl2 200 μM, as previously described.[14,15] The cells were co-transfected with another plasmid containing the construct for the green fluorescent protein (GFP).

Studies were also conducted using a dual incubation of the transiently transfected cells first at 37°C for 24 h and then at 28°C for the last 24 h to determine whether any smaller current densities identified could be rescued, implying a trafficking defect.

2.10 Experimental data and statistical analysis

Electrophysiological data were analysed and plotted using a combination of Clampfit 9.2 and Origin 6.1. If only two groups were compared, we used the unpaired Student's *t*-test. For comparisons among means of more than two groups, analysis of variance was used with *post hoc* pair wise comparisons by Duncan test. Either two-sided $P < 0.05$ or $P < 0.01$ was considered to be statistically significant.

2.11 Ethics statement

Approval for the study was granted by local or university Ethics Review Boards of each participating institution. The study conformed to the Declaration of Helsinki and informed consent was given by all patients for their inclusion.

3. Results

3.1 Study population

Table 1 summarizes the clinical demographics of the 156 unrelated *SCN5A* mutation-negative Type 1 BrS patients. The majority of patients were male ($n = 125$, 80%) and all were of European ancestry with a mean age at diagnosis of 48 ± 14 years. The group was highly symptomatic with 100 individuals (64%) having suffered either prior cardiac arrest or cardiogenic syncope. Nearly half (46%) had a spontaneous type 1 ECG. Approximately half also had a family history of SCD.

3.2 Identification of putative pathogenic mutations

The coding regions and intron/exon boundaries of seven genes previously associated with QRS duration (*SCN10A*, *HAND1*, *CASQ2*, *TKT*, *PLN*, *TBX5*, *TBX3*) were sequenced. Overall, seven of 156 patients (4.5%; seven males) had novel putative pathogenic variants based on the synergistic use of a number of *in-silico* prediction tools,[7] and the absence of the variant in 4300 European ancestry controls from the NHLBI GO ESP (*Table 2* and Supplementary material online, *S3*).

These variants included four potentially pathogenic NS variants in *SCN10A:* E19K (rs141810266) had previously only been seen in the African-American general population (ESP AA MAF 0.07%); R1121C was novel and present at the same codon as two previously observed

Table 2 Novel mutations, absent in 8600 Caucasian reference alleles from the NHLBI GO ESP and rare non-synonymous genetic variants (present in published and internal controls with a measurable frequency <1%)

Gene-variant	rsID	Predicted classification	Risk/ non-risk alleleles	Genotype frequencies						Risk allele frequency		*P*-value
				Cases			ESP Controls			Cases	ESP Controls	
				Hom	Het	Hom (risk)	Hom	Het	Hom (risk)			
SCN10A												
E19K	rs141810266	pathogenic	A/G	155	1	0						
A200V[a]	novel	pathogenic	T/C	155	1	0						
R1121C	novel	pathogenic	T/C	155	1	0						
G1299A	novel	pathogenic	C/G	155	1	0						
I671V	novel	benign	A/G	155	1	0						
G590R	rs35332705	benign	T/C	155	1	0						
TKT												
R148Q	novel	pathogenic	A/G	155	1	0						
S427C	novel	pathogenic	G/C	155	1	0						
TBX5												
G145R	novel	pathogenic	A/G	155	1	0						
PLN												
R25C	novel	benign	C/T	155	1	0						
SCN10A												
G1523Y	rs142217269	pathogenic	C/T	155	1	0	4277	23	0	0.003	0.003	0.5776
S1337T	rs11711062	benign	A/T	155	1	0	4254	46	0	0.003	0.005	0.9018
V1287I	rs145032037	pathogenic	C/T	155	1	0	4289	11	0	0.003	0.001	0.3499
R1268Q	rs138832868	benign	C/T	155	1	0	4276	24	0	0.003	0.003	0.5925
W1139C	rs143744796	pathogenic	C/G	155	1	0	4298	2	0	0.003	0.000	0.102
R14L	rs141207048	pathogenic	C/A	155	1	0	4274	26	0	0.003	0.003	0.9592
CASQ2												
D310N	rs141314684	benign	C/T	155	1	0	4295	5	0	0.003	0.001	0.5225
TKT												
F393L	rs138820989	benign	A/G	153	3	0	4282	18	0	0.010	0.002	0.037

[a]Although absent in 8600 reference alleles from the NHLBI ESP GO study, two individuals within UK10K were heterozygous for this variant.

rare substitutions [R1121H (rs201588811) and R1121S (rs146965005)]; G1299A which was absent in ESP; and A200V which although absent in the larger ESP study, was later identified in two individuals from the UK10K study. Two novel NS *TKT* variants were observed (R148Q and S427C), and a novel NS variant in *TBX5* (G145R).

Three additional novel NS variants were identified, which were not classified as pathogenic, but were absent in the ESP Caucasian controls: *SCN10A* I671V (found in the same individual carrying *SCN10A* G1299A), *SCN10A* G590R, and *PLN* R25C (*Table 2*).

3.3 Rare genetic variants

In addition to these 10 novel variants, eight rare NS genetic variants present in published and internal controls with a frequency <1% were identified (*Table 2*). The minor allele frequencies (MAF) of these eight variants (including four in *SCN10A* predicted as pathogenic—G1523Y, V1287I, W1139C, R14L) did not differ between BrS cases and controls from the NHLBI GO ESP (nor with UK10K, data not shown). There was a suggestive difference in MAF for the *TKT* F393L variant (BrS 1% vs. EVS 0.2%, $P = 0.037$); this variant was predicted to be benign.

Overall, we identified 12 rare NS *SCN10A* variants in 11 of the 156 BrS probands screened, leading to a positive proband yield of 7%. This is lower, however, (8/156−5%) when taking into account only the NS variants predicted as pathogenic.[7] Rare NS variation in *SCN10A* was present in ESP controls at a frequency of 2.65% (102 rare NS variants, five frameshift, two deletion, and ive nonsense variants, in 4300 European ancestry individuals) and in UK10K at a frequency of 2.74% (44 rare NS variants and four nonsense variants, in 1754 individuals sequenced). The signal-to-noise ratio for rare variants in *SCN10A* was therefore 2.6:1. *Figure 1* depicts the positions of novel and rare variants found in *SCN10A* in BrS in the Nav1.8 protein topology.

3.4 Rare-variant association analysis

The results of our collapsed rare variant association test for *SCN10A* are shown in Supplementary material online, *Table S4*. We collapsed uncommon [1−5%] and rare variants [<1%] separately to clearly show

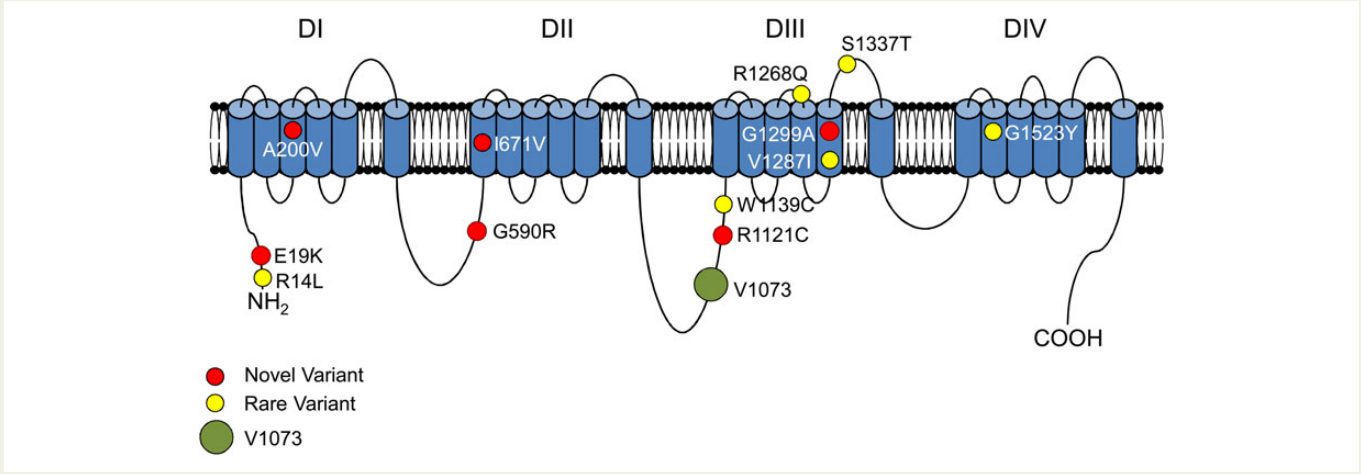

Figure 1 Representation of Nav1.8 channel protein and the corresponding localization of 12 rare variants of which six were novel (E19K, A200V, I671V, G590R, R1121C, G1299A) and the common BrS associated SNP (V1073).

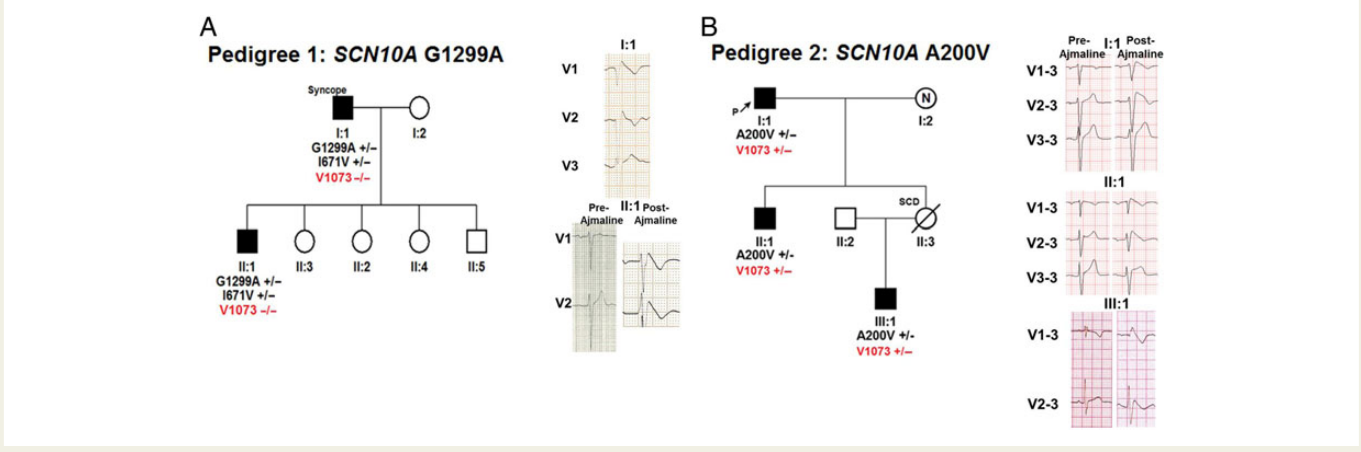

Figure 2 Pedigree 1 demonstrates the *cis* inheritance of *SCN10A* I671V and G1299A in father (I:1) and son (II:1). The ECGs shows a spontaneous type 1 ECG pattern in lead V1 in (I:1) and an ajmaline-induced type 1 pattern in leads V1 and V2. Pedigree 2 shows the failure of the A200V mutation to segregate with phenotype. The ECGs demonstrate the type 1 pattern in all three individuals in the 3rd intercostal space after ajmaline provocation. SCD, sudden cardiac death; V1-3, V2-3, and V3-3 are leads V1–V3 displaced to the 3rd intercostal space.

their contributions using the SKAT and SKAT-O methods. None of the tests revealed a significant association with BrS.

3.5 Common genetic variants

We identified a number of common (>1%) synonymous variants in *TKT* (P194P, D155D, A365A, and P598P), and *HAND1* (S156S, R177R), and for *SCN10A*, we identified 10 synonymous common variants and seven NS common variants (Supplementary material online, *Table S5*).

3.6 Common-variant analysis

We carried out a case–control association analysis for the *SCN10A* common variants using the UK10K control m($n = 1279$) (*Table 3*). Of these common variants, the NS variant V1073A (rs6795970) (A>G; rs6795970) was the most strongly associated SNP with a considerably higher MAF (encoding the Valine and annotated as V1073) of 66.9% in BrS compared with 40.1% in UK10K controls ($P = 8.07 \times 10^{-19}$; odds ratio [OR] [CI 95%]: 3.02 [2.35–3.87]). After conditional analysis adjusting for this variant, no other associations remained. The rs6795970 SNP appears in databases as a Valine to Alanine substitution at position 1073. However, the ancestral allele for rs6795970 and the major allele in ESP and UK10K controls is a G (MAF = 59.9%) encoding the Alanine residue (annotated here as A1073). V1073 is also in strong linkage disequilibrium (LD) with an intronic SNP, rs10428132 ($r^2 = 0.97$), the lead SNP identified in the Brugada GWAS[4] and associated with PR interval and QRS duration in the general population.[16–19] Indeed in controls, rs6795970 was associated with significantly increased PR interval ($P = 8.36 \times 10^{-8}$, $\beta = 4.9$, SE = 0.91) and QRS duration ($P = 2.90 \times 10^{-3}$, $\beta = 1.2$, SE = 0.40), although in cases this was only a trend ($P = 0.5$, $\beta = 2.1$, SE = 3.1; and $P = 0.11$; $\beta = 3.3$; SE = 2.1, respectively).

3.7 Clinical pedigree analysis

Novel putative pathogenic variants were further validated by genotyping available relatives of each proband from four out of a possible seven families, and assessing segregation with BrS phenotype:

Table 3 Common SNPs in *SCN10A* associated with BrS

Gene-variant	rsID	Predicted classification	Minor/major allele[a]	Genotype frequencies						Minor allele frequency[a]		Odds ratio (95% CI)	*P*-value
				Cases			UK10K Controls			Cases	Controls		
				Hom (minor)	Het	Hom (major)	Hom (minor)	Het	Hom (major)				
T1131T	rs6771157	benign	C/G	3	27	126	65	489	724	0.106	0.242	0.37 (0.25–0.54)	3.82×10^{-08}
L1092P	rs12632942	benign	G/A	3	28	125	66	489	724	0.109	0.243	0.38 (0.26–0.55)	7.81×10^{-08}
V1073A	**rs6795970**	**benign**	**A/G**	**74**	**58**	**22**	**200**	**627**	**453**	**0.669**	**0.401**	**3.02 (2.35–3.87)**	**8.07×10^{-19}**
T1064T	rs6791171	benign	T/C	1	14	141	23	351	905	0.051	0.155	0.29 (0.18–0.49)	5.31×10^{-07}
G979G	rs59468016	benign	A/G	3	26	127	68	479	731	0.103	0.241	0.36 (0.25–0.53)	3.16×10^{-08}
I962V	rs57326399	benign	C/T	3	28	125	68	480	731	0.109	0.241	0.39 (0.27–0.56)	1.32×10^{-07}
L492L	rs7617919	benign	A/G	6	18	132	65	461	753	0.096	0.231	0.35 (0.24–0.52)	5.95×10^{-08}
E428E	rs62244070	benign	T/C	3	27	126	65	457	757	0.106	0.229	0.40 (0.27–0.58)	5.69×10^{-07}

[a]In controls. The most significantly associated SNP, V1073A, is highlighted in bold. NB that the ancestral allele for rs6795970 and the major allele in ESP and UK10K controls is a G (MAF 1/4 59.9%) encoding the Alanine residue.

SCN10A G1299A: this 46-year-old male (I;1) who presented with prior syncope at age 34 and a spontaneous type 1 ECG pattern was also a heterozygous carrier for the *SCN10A* I671V. He was treated with an ICD implant and hydroquinidine. His asymptomatic 19-year-old son (II:1) was positive for the type 1 pattern after ajmaline provocation. He carried the same pair of variants confirming a *cis* pattern of inheritance. No other family members were assessed (*Figure 2*).

SCN10A A200V: the carrier was a 58-year-old male (I:1) whose daughter (II:3) had died suddenly in her sleep age 30, with a negative autopsy and toxicology. The father (I:1), his 33-year-old son (II:1), and his grandson (III:1) were all asymptomatic with normal resting ECGs. They were challenged with ajmaline which identified the type 1 pattern in all three. The son (II:1) and grandson (III:1), however, did not carry A200V (*Figure 2*) and two heterozygous carriers were later identified in the UK10K controls. This suggested that A200V was not a monogenic cause for BrS in this family.

The remaining five cases and two families are described in Supplementary material online, *data*.

3.8 Functional assessment

The V1073 and A1073 common variants and two rare variants (A200V and I671V) were assessed for functional effects upon $Na_V1.8$. A200V was thought to be a promising candidate mutation prior to segregation studies and availability of UK10K data. Site-directed mutagenesis for G1299A, found in *cis* with I671V, was unable to produce a sufficiently stable plasmid to permit transfection.

Voltage-clamp experiments were performed following transient expression in ND7/23 cells. As *SCN10A* A1073 is protective for BrS, it was used as an arbitrary comparator for the rest of the *SCN10A* variants that were characterized electrophysiologically. *Figure 3* shows representative whole-cell current traces obtained for A1073 (*A*), V1073 (*B*), and the two rare variants A200V and I671V (*C* and *D*). In all cases, the currents displayed slow kinetics, with a time-to-peak (TTP) $\sim 4-5$ ms and peak activation between $+10$ and $+20$ mV. Compared with A1073, V1073 displayed a lower peak I_{Na} current with A200V and I671V displaying even more marked loss-of-function phenotypes (*Table 4* and *Figure 3*). Both rare variants and V1073 exhibited a shift to the left (more negative voltages) for both V1/2 activation and V1/2 inactivation when compared with A1073 (*Figure 4*). Supplementary material online, *Figure S1* depicts the normalized currents recorded at $+10$ mV for V1073. We measured the late I_{Na} for all variants 100 ms after the triggering stimulus was applied. Due to its slower kinetics, under normal conditions V1073 current exhibits a smaller late I_{Na} when compared with other faster sodium channels like $Na_V1.5$.[14] This I_{Na-L} represents on average $\sim 8\%$ of the peak I_{Na} of the V1073 current. A1073 and both rare variants displayed much larger late currents than the V1073 channel (*Table 4*).

3.9 Trafficking studies

There was no effect of decreased temperature on peak currents for V1073, A200V, and I671V transfected cells (Supplementary material online, *Figure S2*). Thus we cannot conclude whether the decreased currents are due to mistrafficking (not corrected by low-temperature incubation) or alternate mechanisms.

4. Discussion

This manuscript describes the multi-centre investigation of 156 *SCN5A* mutation-negative BrS probands for genetic variation associated with loci previously shown to influence the QRS duration. The majority

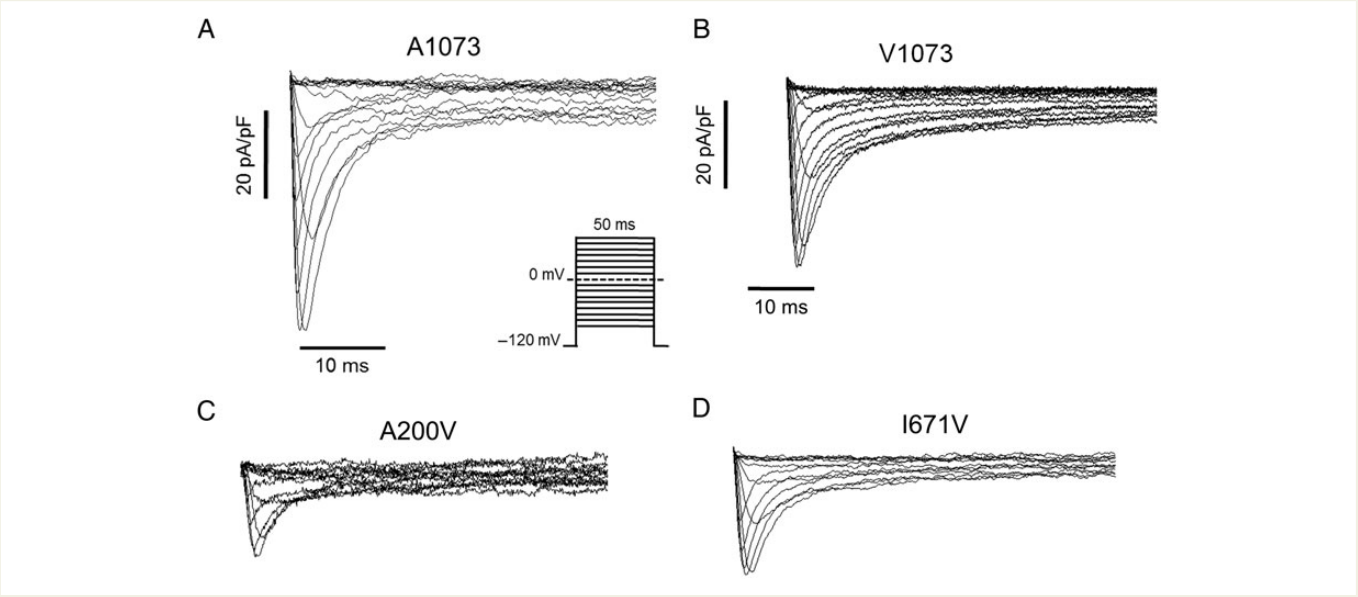

Figure 3 Representative traces obtained for the *SCN10A* common variants A1073 and V1073, and rare variants A200V and I671V transiently expressed in ND7/23 cells ($n = 5$ cells/group). Inset in (*A*) represents the voltage-clamp protocol.

Table 4 Biophysical properties of *SCN10A* variants A1073, BrS associated SNP V1073, and rare *SCN10A* variants A200V and I671V transiently expressed in ND7/23 cells (data are expressed as mean ± SE)

	A1073	V1073	A200V	I671V
Peak I_{Na} (pA/pF)	−57.4 ± 5.8	−40.0 ± 3.1*	−17.9 ± 2.7**	−27.1 ± 5.9**
V at I–V peak (mV)	28.8 ± 1.3	6.7 ± 1.9***	11.7 ± 3.1***	10.0***
$V_{1/2}$ activation (mV)	−6.3 ± 0.9	−18.0 ± 3.0**	−12.9 ± 1.2**	−15.8 ± 0.7**
$V_{1/2}$ inactivation (mV)	−53.8 ± 1.8	−75.6 ± 2.7**	−59.2 ± 1.0*	−68.9 ± 1.0**
τ Fast (ms)	2.3 ± 0.2	2.9 ± 0.2	2.6 ± 0.4	3.1 ± 0.3*
τ Slow (ms)	9.3 ± 0.8	21.8 ± 2.4***	22.6 ± 3.2***	30.6 ± 1.7***
$I_{Na\text{-}L}$ (% peak I_{Na})	16.1 ± 1.7	7.8 ± 1.3**	25.4 ± 2.6*	16.3 ± 2.9
Cells (*n*)	7	9	6	6

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

was symptomatic and most had a family history of BrS or SCD suggesting a rich cohort for detection of novel genetic signals. Despite this, only a small number [18 of 156 (11.5%)] of BrS cases carried rare (MAF <1%) NS variants. These were mainly found in *SCN10A* [12 of 18 (67%)]. Putative pathogenicity was predicted by the synergistic use of four commonly used *in silico* prediction tools (GERP, Grantham, SIFT and Polyphen2) as in isolation their specificities are low particularly when applied to LQTS genetic testing.[7] This led to eight of 12 rare NS variants in *SCN10A* being classified as putative pathogenic and a positive proband yield of 5.1%, considerably lower than that typically found for mutations in *SCN5A* (20%). The signal-to-noise ratio was (2.6:1) when comparing our BrS cases to ESP controls. This is much lower than that for *SCN5A* which is typically (10:1).[20] Furthermore, we found no evidence for rare *SCN10A* variants in aggregate being associated with BrS using SKAT. When our pedigrees were assessed, only the pair of variants in *cis* (G1299A and I671V) co-segregated with the phenotype in an affected father and affected son. Our data suggest that rare variation in *SCN10A*, particularly in *SCN5A* mutation negative cases, is unlikely to cause BrS. This contrasts markedly with a recent paper by Hu *et al.*[21] which identified *SCN10A* mutations in 16.7% of 150 BrS probands although a signal-to-noise ratio or gene-burden analysis was not provided. This difference in yield cannot be explained from a technical perspective as conventional Sanger sequencing was undertaken in both studies. Of note, Hu *et al*. studied only 200 ethnically matched controls without finding any missense rare variants. This is unsurprising as ESP and UK10K data both show that there are plenty of rare variants in controls but larger numbers are required to detect them reliably. In addition, all bar four out of 17 putative mutations detected by Hu *et al*. were present in ESP and one of these four was also seen in 1000Genomes. While a novel frameshift mutation was found in the Hu cohort, many nonsense mutations were also present in both ESP and UK10K cohorts. Therefore, the prevalence of novel rare variants is actually only three out of 150 (2%) compared with seven of 156 (4.5%) in ours. The profile of our population also differed from Hu *et al.*'s primarily due to the inclusion

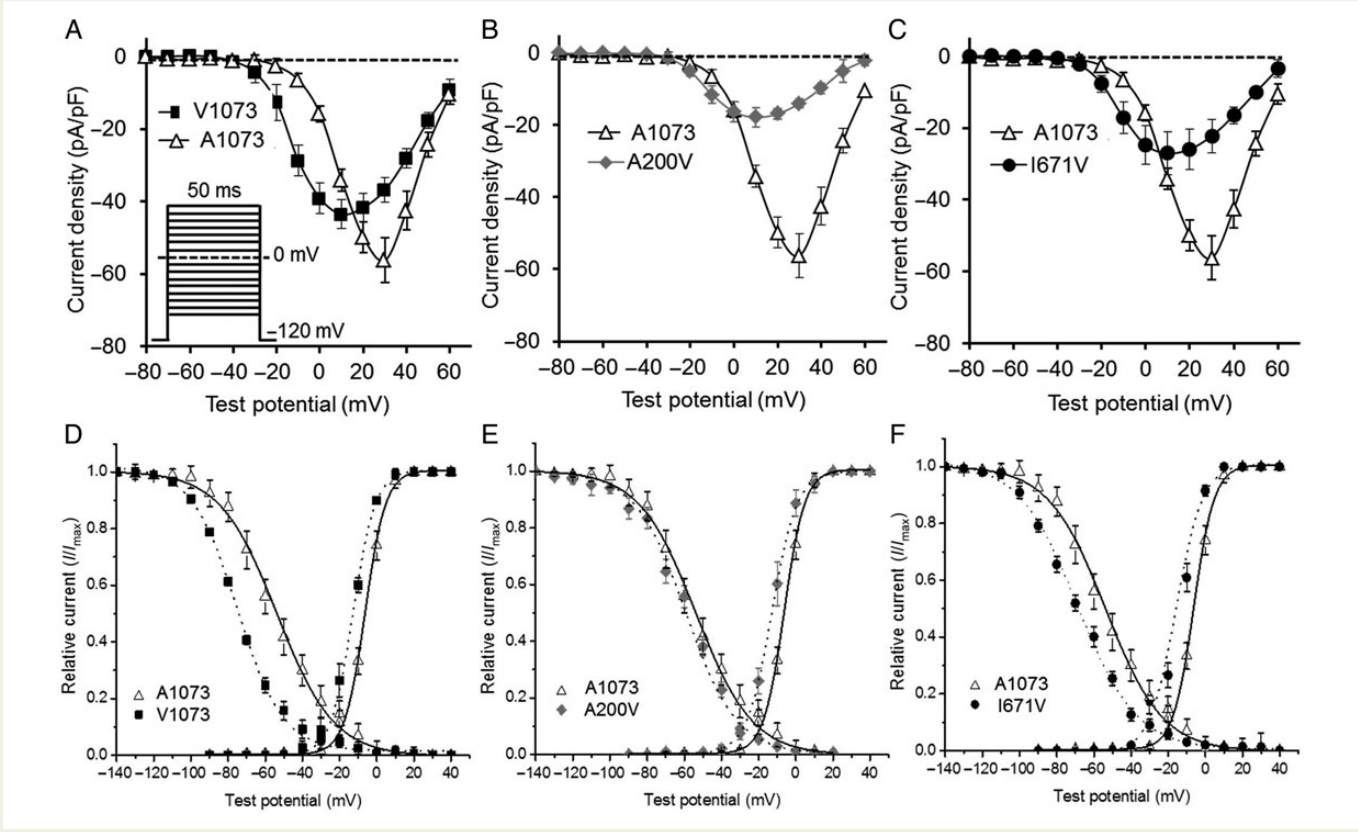

Figure 4 (*A*–*C*) Current–voltage relationships obtained for *SCN10A* common variants A1073 and V1073, and rare variants A200V and I671V expressed in ND7/23 cells ($n = 6$–9 cells/group). Inset in (*A*) shows the representation of the voltage-clamp protocol used. (*D*–*F*) Voltage-dependence of activation and inactivation for studied variants expressed in ND7/23 cells. The data are presented as peak currents normalized by the peak I_{Na} obtained at test potential = +10 mV. The average data for the A1073 common variant is presented in each panel for comparison. Data are expressed as mean ± SE.

criteria of negative *SCN5A* genotype (100% vs. Unspecified, respectively) and higher prevalence of a family history of SCD (47 vs. 19%, respectively). Both factors would suggest a greater likelihood of finding pathogenic *SCN10A* mutations. Thus our 'enriched' cohort and more stringent 'mutation' definition are more likely to be representative of the yield of novel rare *SCN10A* variants in BrS.

We did, however, find strong association of common and functional genetic variation in *SCN10A* with BrS. V1073 was strongly associated with BrS (66.9%) compared with controls (40.1%), and significantly increased PR and QRS duration in controls. This variant is in tight LD with rs10428132, the lead SNP in the BrS GWAS and in GWAS for PR interval and QRS duration.[3,16–19] It is only 640 bp away from the *SCN5A-SCN10A* enhancer and TBX3/5 binding site which encompasses the common intronic functional variant rs6801957 ($r^2 = 0.933$ between rs6795970 and rs6801957).[22]

4.1 Functional effects of *SCN10A* variation

Due to A1073 (rs6795970) being protective for BrS, we chose it as an arbitrary comparator. We observed diminished peak I_{Na} in BrS associated variants although V1073 was more mildly impaired than I671V and A200V. We could not determine whether this was due to defective trafficking. This will require additional experiments such as protein-expression studies. The co-segregation of G1299A/I671V with BrS in one pedigree supports the hypothesis that functional effects of rare *SCN10A* variants such as I671V may be contributory to the BrS phenotype. Unfortunately, a lack of co-segregation in the A200V pedigree detracts from this possibility.

Thus interpretation of variants of unknown significance represents a major diagnostic challenge. Despite the synergistic use of four *in silico* tools, predicted pathogenicity in *SCN10A* did not always correspond to disease association (A200V). This demonstrates the potential unreliability of these methods, particularly if variants are classified as benign and yet have functional effects (I671V and even common variant V1073).

Interestingly, although A1073 appears to be protective for BrS, it has a larger $I_{Na\text{-}L}$ compared with V1073 but is of similar magnitude when compared with the rare variants. This might be related to a heterologous expression system such as the ND7/23 cell line allowing only a partial characterization of currents and may represent a limitation of the study. Alternatively, if functional effects of genetic variation in *SCN10A* are important in BrS, $I_{Na\text{-}L}$ may not be responsible.

It is uncertain whether the diminished peak $Na_V1.8$ current associated with V1073 impacts directly upon cardiac conduction and the BrS phenotype or leads to the disruption of the *SCN5A-SCN10A* enhancer and TBX3/5 binding site as proposed by van den Boogaard *et al.*[22] Data from an *SCN10A* knockout mouse model and electrophysiological data from Na_V 1.8 in the rat suggest[14,17] that a direct functional effect may be present but human data are sorely lacking. The selective $Na_V1.8$ blocker, lacosamide, is used in patients with epilepsy and neuropathic pain and is associated with mild increases in PR interval. Pre-clinical data in dogs have also identified prolongation of the PR interval and QRS duration

during therapy (http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_Product_Information/human/000863/WC500050338.pdf). In the mouse, however, $Na_V1.8$ is expressed in the neuron and not ventricular cardiomyocytes and $Na_V1.8$ blockade affects neuronal electrophysiology only.[23] Hu *et al.*[21] presumed significant $Na_V1.8$ expression in human ventricular myocardium and over-expressed it in a heterologous system in a 1:1 ratio with $Na_v1.5$ to demonstrate functional effects. This level of co-expression is unproved in the human cardiomyocyte[22] and indeed the existence of a Nav1.8 and Nav1.5 complex *in vivo* is unclear as co-immunoprecipitation was not undertaken in human myocardium. For these reasons, we did not undertake co-expression studies ourselves.

5. Conclusions

Rare variation in several candidate genes arising from the QRS GWAS does not appear to associate significantly with BrS. Our data do not support a strong role for *SCN10A* causing monogenic BrS despite experimental data for two rare *SCN10A* variants demonstrating a loss of function. The *SCN10A* NS SNP V1073 was, however, strongly associated with BrS and also demonstrated loss of function. Further research is required to determine whether this may be due to a direct functional effect in neurons or cardiac tissue or reflect disruption of *SCN5A* transcription in cardiomyocytes. A clinical role for *SCN10A* genotyping cannot be recommended.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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Conflict of interest: M.J.A. is a consultant to Boston Scientific, Gilead Sciences, Medtronic and St Jude Medical and has intellectual property in and receives royalties from Transgenomics (FAMILION). A.W. is a consultant to Sorin. J.J. is now an employee of Leo Pharma (A/S), Denmark and A.G.H. is an employee of Novo Nordisk (A/S), Denmark.

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