**RRAD mutation causes electrical and cytoskeletal defects in cardiomyocytes derived from a familial case of Brugada syndrome**

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**Aims**

The Brugada syndrome (BrS) is an inherited cardiac disorder predisposing to ventricular arrhythmias. Despite considerable efforts, its genetic basis and cellular mechanisms remain largely unknown. The objective of this study was to identify a new susceptibility gene for BrS through familial investigation.

**Methods and results**

Whole-exome sequencing performed in a three-generation pedigree with five affected members allowed the identification of one rare non-synonymous substitution (p.R211H) in **RRAD**, the gene encoding the RAD GTPase, carried by all affected members of the family. Three additional rare missense variants were found in 3/186 unrelated index cases. We detected higher levels of **RRAD** transcripts in subepicardium than in subendocardium in human heart, and in the right ventricle outflow tract compared to the other cardiac compartments in mice. The p.R211H variant was then subjected to electrophysiological and structural investigations in human cardiomyocytes derived from induced pluripotent stem cells (iPSC-CMs). Cardiomyocytes derived from induced pluripotent stem cells from two affected family members exhibited reduced action potential upstroke velocity, prolonged action potentials and increased incidence of early afterdepolarizations, with decreased Na\(^+\) peak current amplitude and increased Na\(^+\) persistent current amplitude, as well as abnormal distribution of actin and less focal adhesions, compared with intra-familial control iPSC-CMs. Insertion of p.R211H-**RRAD** variant in control iPSCs by genome editing confirmed these results. In addition, iPSC-CMs from affected patients exhibited a decreased L-type Ca\(^{2+}\) current amplitude.

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Introduction

The Brugada syndrome (BrS) is a rare inherited disorder characterized by a specific but labile pattern at the electrocardiogram (ECG), i.e. a coved ST-segment elevation followed by a negative T wave in the right precordial leads, revealing electrical dysfunctions that predispose to ventricular arrhythmias and sudden cardiac death (SCD).\(^1,2\) Despite numerous studies over the last 25 years, the molecular basis for the ECG abnormalities and the mechanism underlying ventricular arrhythmias and SCD in the context of BrS are still incompletely understood.

Mutations in the SCN5A gene, which encodes the main cardiac Na\(^+\) channel α-subunit Nav1.5, are found in 20–25% of BrS probands.\(^3,4\) These mutations result in a reduction of the cardiac Na\(^+\) current (\(I_{Na}\)) and are thought to decrease rapid cardiomyocyte depolarization and cardiac conduction, a mechanism that may play a key role in BrS pathogenesis.\(^5\) Other rare variants have been reported as predisposing to BrS, affecting more than 20 genes encoding proteins either involved in Nav1.5 regulation, or encoding L-type Ca\(^{2+}\) channel subunits and K\(^+\) channels subunits mainly involved in \(I_{KATP}\) and \(I_{to}\) generation.\(^6,7\) However, these additional variants account for <5% of cases, bringing the molecular diagnosis success rate to only 25–30% of cases.

Apart from SCN5A, the genes associated with BrS susceptibility have been identified only in few unrelated patients or in small families and often through candidate gene approaches: there is increasing evidence that they may play only a minor role, if any, in the phenotype of the patients.\(^8-10\) Moreover, extended familial genetic investigations revealed numerous occurrences of phenocopies and non-penetrance for the identified familial SCN5A variants in families affected by BrS, questioning the autosomal dominant transmission pattern initially proposed.\(^11\) Recently, the identification of three risk loci for BrS by genome-wide association study confirmed a more complex pattern of disease transmission.\(^12\) Despite this genetic complexity, studies on familial cases remain useful to discover new genes involved in disease susceptibility and understand better the pathogenesis of BrS.

In the present study, by applying a whole-exome sequencing approach to a large pedigree with BrS and documented history of SCD, we identified a rare non-synonymous variant (p.R211H) in the RRAD gene, which encodes the RAD (Ras Associated with Diabetes) GTPase.\(^13\) We then demonstrated RAD involvement in BrS pathogenesis based on functional studies in cardiomyocytes differentiated from induced pluripotent stem cells of the family proband. A small but significant excess burden of rare missense variants in RRAD was found in unrelated BrS patients, suggesting that RRAD may be a new susceptibility gene for BrS.

Conclusion
This study identified a potential new BrS-susceptibility gene, RRAD. Cardiomyocytes derived from induced pluripotent stem cells expressing RRAD variant recapitulated single-cell electrophysiological features of BrS, including altered Na\(^+\) current, as well as cytoskeleton disturbances.

Keywords
Brugada syndrome • RAD GTPase • Induced pluripotent stem cells • Sodium current • L-type calcium current • Actin cytoskeleton

Translational perspective
The Brugada syndrome (BrS) is an inherited cardiac disorder predisposing to ventricular arrhythmias and sudden cardiac death. In a large family affected by BrS, we identified a rare missense mutation in RRAD, the gene encoding the RAD GTPase. The mutation leads to electrical and structural defects consistent with BrS in cardiomyocytes differentiated from induced pluripotent stem cells of the family proband. A small but significant excess burden of rare missense variants in RRAD was found in unrelated BrS patients, suggesting that RRAD may be a new susceptibility gene for BrS.
nocturnal agonal respiration. After implantable cardioverter-defibril-lator implantation, neither recurrent symptoms nor ventricular arrhythmias have been observed. During familial screening, four relatives were identified as affected after flecainide challenge (Figure 1B). In addition, individual III:8 exhibited a BrS Type II ECG pattern under flecainide (Supplementary material online, Figure S1). None of these individuals had presented relevant symptoms. However, an unex-plained sudden death occurred at rest in a 41-year-old proband’s uncle (individual II:3). Electrocardiogram and clinical parameters for this French family are provided in Table 1.

**Identification of a rare RRAD variant in a familial case of Brugada syndrome**

Whole-exome sequencing on the index case unveiled 217 rare coding variants (Supplementary material online, Methods). After analysing
variants located in genomic regions shared by all affected family members and co-segregation analyses, six missense variants were detected in all five affected patients (Figure 1C). They were located in RRAD on chromosome 16, H6PD on chromosome 1, and SNAP23 and SPTBN5 (two variants) on chromosome 15 (Supplementary material online, Table S1). While there were six non-affected carriers for each variant on chromosomes 1 and 15, both variants located on chromosome 16 (in RRAD and FHOD1) were carried by only three non-affected relatives (Supplementary material online, Table S1; Figure 1B); the individual III:8 presenting with a BrS Type II ECG pattern, and two women (II:2 and III:4) for whom drug challenge could not be performed. Furthermore, among the six missense variants, the RRAD variant (p.Arg211His) was the only one predicted to be damaging by SIFT and PolyPhen-2 tools (Supplementary material online, Tables S1 and S2). This variant is also associated to the highest CADD score (CADD PHRED score: 33) and is currently reported as the least frequent in the gnomAD database (1/242 446 alleles; Supplementary material online, Table S1). By screening the four coding exons of RRAD in 186 unrelated BrS patients using Sanger sequencing, we identified three additional rare missense mutations in isolated cases: p.Asp46Tyr, p.Gln186Arg, and p.Val215Met (Supplementary material online, Table S2). Overall, we detected a trend for enrichment (P = 0.042, odds ratio = 6.98, 95% confidence interval: 0.79–84.33) in rare RRAD non-synonymous variants (with a MAF below 0.1% in gnomAD) among the BrS cases (three carriers out of 186 cases) compared to a population of 856 reference individuals of French origin, for which RRAD genotype status was available in-house (2 carriers out of 856 individuals; Supplementary material online, Table S2).

Since BrS is characterized by abnormal electrical activity in the right ventricle outflow track (RVOT) region, we compared Rad expression levels in mouse RVOT to its expression in the rest of the right ventricle and the left ventricular free walls. Rad was found four-fold more expressed in the RVOT than in the other ventricular parts (Figure 2A). We also investigated the expression of RAD in human left and right ventricles and observed higher expression in the subepicardium than in the subendocardium for both compartments (Figure 2B).

In order to decipher the electrophysiological consequences of the missense variant in RRAD (and/or possibly in FHOD1) within the family, we differentiated cardiomyocytes from iPSCs obtained from the index case (BrS1; individual III:2; three iPSC clones: BrS101, BrS102, and BrS103) and his unaffected brother (Ctl1; individual III:1; two clones: Ctl103 and Ctl104) who carries the rare variants on chromosome 16.

**Slow rate and reduced action potential upstroke velocity linked to I_{Na} and I_{Ca,L} disruption in Brugada syndrome cardiomyocytes derived from induced pluripotent stem cells**

We first investigated the properties of the ventricular-like action potentials. BrS1 iPSC-CMs displayed slower spontaneous rhythms than Ctl1 iPSC-CMs and lower action potential upstroke velocity (Figure 3A–C). Based on these results, we investigated the biophysical properties of the Na^{+} and L-type Ca^{2+} currents. Membrane capacitance in BrS1 iPSC-CMs was slightly, though not significantly, higher (55.5 ± 25.5 pF, n = 68) than in Ctl1 iPSC-CMs (47.5 ± 22.8 pF; n = 51;
P = 0.07). $I_{\text{Na}}$ density was significantly lower (by ~40% at -25 mV) in BrS1 iPSC-CMs (36.8 ± 16.7 pA/pF) than in Ctl1 iPSC-CMs (58.8 ± 16.5 pA/pF; Figure 3D and E), a result consistent with a lower NaV1.5 protein level in BrS1 iPSC-CMs than in Ctl1 iPSC-CMs (Supplementary material online, Figure S3) while NaV1.5 transcript level was not significantly changed (BrS1: 0.7 ± 0.4, 3 clones; Ctl1: 1.0 ± 0.5, 2 clones). Steady-state activation and inactivation gating properties did not differ between BrS1 and Ctl1 iPSC-CMs but recovery from inactivation was slightly faster in BrS1 iPSC-CMs than in Ctl1 iPSC-CMs (Supplementary material online, Table S4). $I_{\text{CaL}}$ density was also lower (by ~30% at -5 mV) in BrS1 iPSC-CMs (18.9 ± 6.0 pA/pF) compared to Ctl1 iPSC-CMs (26.3 ± 7.1 pA/pF) without any disturbance of activation gating properties (Figure 3F and G; Supplementary material online, Table S4). Similar results have been obtained on iPSC-CMs derived from the proband’s daughter carrying the p.R211H RRAD variant and affected by BrS (BrS2; individual IV:2; Supplementary material online, Figure S4 and Table S4); her unaffected sister was used as control (Ctl2; individual IV:1). No difference in $I_{\text{Na}}$ and $I_{\text{CaL}}$ densities between the three BrS1 iPSC-CM clones and between the two Ctl1 iPSC-CM clones was observed (Supplementary material online, Figure S5).

**Prolonged action potential duration, early afterdepolarizations, and calcium flux disturbances in Brugada syndrome cardiomyocytes derived from induced pluripotent stem cells**

When paced at a cycle length of 700 ms, BrS1 iPSC-CMs showed longer action potentials than Ctl1 iPSC-CMs (Figure 4A and B). No significant difference was observed between the three BrS1 iPSC-CM clones and between the two Ctl1 iPSC-CM clones (Supplementary material online, Figure S6). Interestingly, BrS1
iPSC-CMs also exhibited a larger persistent Na\textsuperscript{+} current than Ctl1 iPSC-CMs (Figure 4C and D). Early afterdepolarizations (EADs) were observed in 80% and 50% of BrS1 iPSC-CMs derived from BrS101 and BrS103 clones, respectively (Figure 4E and F) and in BrS2 iPSC-CMs (Supplementary material online, Figure S4). No EAD was observed in Ctl1 iPSC-CMs and in iPSC-CMs derived from clone BrS102.

Given these results, we investigated intracellular Ca\textsuperscript{2+} flux properties. BrS1 iPSC-CMs exhibited longer [Ca\textsuperscript{2+}], transient decay times than Ctl1 iPSC-CMs (Figure 4G and H), suggesting an impairment of the Ca\textsuperscript{2+} recycling. Moreover, we observed abnormal [Ca\textsuperscript{2+}] oscillations in 40% of the BrS1 iPSC-CMs (Figure 4G). These events are consistent with action potential prolongation and EAD appearance observed in these cells.
Figure 4  Action potential repolarization, persistent Na\(^+\) current (I\(_{\text{NaP}}\)) and intracellular Ca\(^{2+}\) handling in cardiomyocytes derived from induced pluripotent stem cells from the proband (BrS1) and his unaffected brother (Ctl1). (A) Representative action potentials recorded at a pacing cycle length of 700 ms in Ctl1 and BrS1 cardiomyocytes derived from induced pluripotent stem cells. (B) Box plot of action potential duration (APD) at 30% (APD30), 50% (APD50), and 90% (APD90) of full repolarization. Statistical test: two-way analysis of variance with Bonferroni test for multiple comparisons. (C) Representative currents recorded during 1-s voltage-clamp ramps from -120 mV to +40 mV (inset; stimulation frequency: 0.2 Hz) before (Control) and after tetrodotoxin (TTX) perfusion (top traces) in Ctl1 and BrS1 cardiomyocytes derived from induced pluripotent stem cells. Bottom traces show the corresponding TTX-sensitive currents. (D) Box plot of the TTX-sensitive I\(_{\text{NaP}}\) at -20 mV. Mann–Whitney test. (E) Representative examples of early afterdepolarizations recorded in spontaneously beating cardiomyocytes differentiated from BrS101 and BrS103 iPSC clones (BrS1 patient). (F) Early afterdepolarization incidence in the different cardiomyocytes derived from induced pluripotent stem cell lines. \(\chi^2\) test; P values vs. Ctl1. (G) Representative [Ca\(^{2+}\)]\(_i\) transients from Ctl1 and BrS1 cardiomyocytes derived from induced pluripotent stem cells with an example of abnormal Ca\(^{2+}\) oscillations following peak [Ca\(^{2+}\)]\(_i\) transient in BrS cardiomyocytes derived from induced pluripotent stem cells. (H) Box plot of [Ca\(^{2+}\)]\(_i\) transient 75% decay time (Ca\(^{2+}\) transient 75%) in Ctl1 and BrS1 cardiomyocytes derived from induced pluripotent stem cells.
Figure 5 Cytoskeleton in cardiomyocytes derived from induced pluripotent stem cells obtained from the proband (BrS1) and his unaffected brother (Ctl1). (A) Left: representative immunostainings of filamentous actin (F-actin, stained with phalloidin) and troponin I and merged acquisitions with nucleus staining with DAPI in Ctl1 and BrS1 cardiomyocytes derived from induced pluripotent stem cells (top panels) and corresponding fluorescence distribution measured at the level of the yellow dotted lines (fluorescence-distance relationships; bottom panels). Scale bars: 20 μm. Right panel: percentage of Ctl1 (n = 10) and BrS1 (n = 18) cardiomyocytes derived from induced pluripotent stem cells with striated (in white) and cortical (in black) F-actin staining. Statistical test: Fisher’s exact test. (B) Left: representative three-dimensional acquisition illustrations of a Ctl1 and a BrS1 cardiomyocytes derived from induced pluripotent stem cell after immunostaining of F-actin and Troponin I (left panels). Width: 107.12 μm; height: 12 μm; and depth: 107.12 μm. Right panel: box plots of thickness and cell circularity (cell width/cell length ratio) in Ctl1 (n = 10) and BrS1 (n = 12) cardiomyocytes derived from induced pluripotent stem cells Mann–Whitney test.
Cytoskeleton defects in Brugada syndrome cardiomyocytes derived from induced pluripotent stem cells

The BrS1 iPSC-CMs displayed impaired F-actin organization and cortical distribution of troponin I, while Ctl1 iPSC-CMs displayed a striated distribution of both proteins. This cytoskeleton defect was observed in 70% of BrS1 iPSC-CMs but in only 8% of Ctl1 iPSC-CMs (Figure 5A). Three-dimensional views using confocal microscopy showed that Ctl1 iPSC-CMs had flat cell bodies, whereas BrS1 iPSC-CMs exhibited round cell shape making the cell thickness larger than in Ctl1 iPSC-CMs (Figure 5B). In addition, the density of vinculin-containing adhesion complexes was lower in BrS1 iPSC-CMs than in Ctl1 iPSC-CMs, with impaired localization of vinculin (Supplementary material online, Figure 5F). Similar results have been obtained in BrS2 iPSC-CMs (Supplementary material online, Figure 5B).

**RRAD p.R211H knock in isogenic line recapitulates the Brugada syndrome phenotype observed in the Brugada syndrome family members cell lines**

RRAD p.R211H variant was inserted into an extra-familial control iPSC line by CRISPR/Cas9 technology (Supplementary material online, Figure 5A). Action potential recordings showed that genome edited (Rad R211H ins) iPSC-CMs had slower spontaneous rhythms, lower action potential upstroke velocity and longer action potential duration than control (Rad WT) iPSC-CMs (Figure 6A–C). Early afterdepolarizations were observed in 20% of Rad R211H ins iPSC-CMs and in none of the Rad WT iPSC-CMs (Figure 6D). Intracellular calcium recordings showed that Rad R211H ins iPSC-CMs exhibited an even beating rate when compared with Rad WT iPSC-CMs as well as a slowing in the calcium reuptake (Figure 6E and F). Rad R211H ins iPSC-CMs exhibited a significantly lower peak $I_{Na}$ density than Rad WT iPSC-CMs (30.3 ± 14.2 pA/pF vs. 56.8 ± 35.0 pA/pF, respectively, at -25 mV), with no change in steady-state activation and inactivation properties (Supplementary material online, Table S4). Rad R211H ins iPSC-CMs also exhibited a larger persistent Na$^+$ current than RAD WT iPSC-CMs (Figure 6G and H). No appreciable difference between Rad R211H ins iPSC-CMs and Rad WT iPSC-CMs in $I_{CaL}$ density was observed (Supplementary material online, Figure 5F). Finally, as in iPSC-CMs of the family members affected by BrS, the insertion of the p.R211H variant in the extra-familial control line triggered cytoskeleton defects in ~40% of the cardiomyocytes (Supplementary material online, Figure 5C).

In addition, the effects of RRAD p.R211H variant were also investigated in neonatal mouse ventricular myocytes infected with adenoviruses encoding Green Fluorescent Protein (GFP) alone, wild type human RAD plus GFP, or p.R211H-RAD plus GFP. These experiments confirmed the results obtained with iPSC-CMs (Supplementary material online, Results).

The p.R211H variant triggers a gain of function effect on RAD GTPase activity

To directly assess the impact of the p.R211H variant on RAD activity, we performed nucleotide exchange kinetics measurements on WT-RAD and R211H-RAD proteins. As no guanine exchange factor is described for RAD GTPase, kinetics were initiated by Mg$^{2+}$ chelation with ethylenediaminetetraacetic acid (EDTA) R211H-RAD protein showed an increased activity compared to WT-RAD (Figure 7A and B) with Kobs values that were significantly higher (respectively 0.083 ± 0.025 s$^{-1}$ and 0.03 ± 0.008 s$^{-1}$). These results suggest that the variant has a direct positive effect on RAD GTPase activity, consistent with a gain of function of the protein.

**Discussion**

In this study, we identified a novel BrS-susceptibility gene, RRAD, and showed that the RRAD variant identified in a familial form of the disease leads to a BrS-typical electrical defect, i.e. reduced amplitude of $I_{Na}$, coupled with cardiomyocyte cytoskeletal abnormalities. At the cellular level, such dual effect has never been reported in the context of this hereditary arrhythmia.

In the past two decades, BrS has been associated to more than 20 susceptibility genes.14 Most of those genes, including SCN5A, have been identified using functional candidate approaches, with only few mutations co-segregating with BrS ECG anomalies in familial forms. In the present study, we applied a hypothesis-free approach, based on whole-exome sequencing and identity-by-descent analysis, to a familial case with genetically unexplained BrS. Following this strategy, we identified six missense variants shared by the five family members exhibiting the BrS phenotype. Among these variants, the p.R211H substitution in the RRAD gene was (i) the least frequent in public databases (reported in only one case), (ii) the only variant predicted as deleterious by both SIFT and Polyphen-2, and (iii) the variant with the highest CADD score. These results indicate that p.R211H is the most likely causal variant in this familial case, independently of the biological function of RRAD gene product.

We then tested the relative contribution of RRAD variants in BrS susceptibility by screening for rare non-synonymous variants within its sequence among unrelated index cases compared to reference individuals, all of French origin. We observed a trend for enrichment in rare non-synonymous variants among cases (P < 0.05)—with 3/186 affected individuals carrying rare missense mutations vs. 2/856 reference individuals—thus strengthening the likelihood of RRAD involvement in BrS pathophysiology.

The RAD protein is a member of the RGK subfamily of Ras GTPases that has previously been associated with ventricular arrhythmias in mice.14 Here, we showed higher levels of RRAD protein in subepicardium than in subendocardium in human heart, as well as predominant expression of Rad in the right ventricle outflow tract compared to the other cardiac compartments in mice: both expression patterns are fully concordant with a role in BrS pathogenesis.6

Results obtained with iPSC-CMs demonstrate that the RRAD p.R211H variant reduces $I_{Na}$ by about 40%. To our knowledge, this is the first report of an effect of RAD on the cardiac Na$^+$ channel. The involvement of the RRAD p.R211H variant in $I_{Na}$ down-regulation was confirmed by inserting the mutation by genome editing in a control human iPSC line obtained from a healthy individual unrelated to the family and by expressing the mutated protein in neonatal mouse ventricular cardiomyocytes in primary culture. The studies performed on this second model suggest that the p.R211H variant leads to a gain
Figure 6 Rad p.R211H insertion in an extra-familial control line by genome editing: electrophysiological characterization. (A) Representative action potential recordings from control (Rad WT) and mutated (Rad WT) conditions. (B) Box plots of peak to peak duration and action potential upstroke velocity (dV/dt max) of Rad WT and Rad WT cardiomyocytes derived from induced pluripotent stem cells Mann–Whitney test. (C) Mean action potential duration (APD) at 30% (APD30), 50% (APD50), and 90% (APD90) of full repolarization at a pacing cycle length of 1 s. Two-way ANOVA with Bonferroni test for multiple comparisons. (D) Early afterdepolarization incidence in Rad WT and Rad R211H ins cardiomyocytes derived from induced pluripotent stem cells. (E) Representative [Ca2+]i transients obtained in Rad WT and Rad R211H ins cardiomyocytes derived from induced pluripotent stem cells and their corresponding fluorescence map. (F) [Ca2+]i transient 75% decay time (Ca2+ transient 75%) in Rad WT and Rad R211H ins cardiomyocytes derived from induced pluripotent stem cells Mann–Whitney test. (G) Superimposed representative I_{Na} traces recorded in Rad WT and Rad R211H ins cardiomyocytes derived from induced pluripotent stem cells (upper panel; voltage-clamp protocol in inset) and mean (±SEM) current density-membrane potential relationships (bottom). *P < 0.05, **P < 0.01, and ***P < 0.001 (two-way analysis of variance with Bonferroni post hoc test for multiple comparisons). (H) Top: representative currents recorded during 1-s voltage-clamp ramps from -120 mV to +40 mV (stimulation frequency: 0.2 Hz) before (Control) and after tetrodotoxin (TTX) perfusion (top traces) in Rad WT and Rad R211H ins cardiomyocytes derived from induced pluripotent stem cells and corresponding TTX-sensitive persistent sodium current (I_{NaP}, bottom traces). Bottom: box plots of I_{NaP} density. Mann–Whitney test.
of function of RAD. Indeed, its effect on $I_{\text{Na}}$ is mimicked by a 10-fold overexpression of wild-type RAD and at this level of expression, both the WT and the p.R211H RAD have the same effect on $I_{\text{Na}}$. This gain-of-function hypothesis is supported by the two-fold faster GDP/GTP exchange activity of RAD that we found in the mutant protein compared to the WT RAD. Interestingly, the arginine-211 residue is highly conserved among species and among the RGK protein members. It is localized between the G4 and the G5 sites of the RAD guanine recognition area located in the G domain and may play an important role on RAD affinity for GTP. Our results suggest that at least part of p.R211H RAD effects on $I_{\text{Na}}$ involves a down-regulation of Nav1.5 expression at the protein level, without any alteration of SCN5A transcription. Further studies will be needed to elucidate the mechanisms of this down-regulation and whether RAD also interacts with Nav1.5 or one of its regulatory subunits to directly modulate its function.

**Take home figure** The p.R211H mutation in the gene encoding RAD GTPase is involved in Brugada syndrome pathogenesis, leading to both electrical and structural defects in cardiomyocytes differentiated from induced pluripotent stem cells of affected patients.
The iPSC-CMs of the mutated patients and the genome edited Rad R211H ins iPSC-CMs were also characterized by the presence of a large persistent Na\(^+\) current and the occurrence of EADs. The effects of RRAD p.R211H variant on the Na\(^+\) current, i.e. a decrease in Na\(^+\) peak current amplitude and the occurrence of a persistent Na\(^+\) current, are similar to those produced by the SCNsA mutation p.1795insD, which causes an overlap syndrome combining the phenotypic traits of BrS and Type 3 long QT syndrome.\(^{16}\) However, mouse studies suggest that RAD is preferentially expressed in the RVOT which could limit the effects of the mutation to this specific area, which is insufficient to trigger appreciable QT prolongation. This would explain why the RRAD p.R211H carriers exhibit normal QT intervals.

At first glance, EADs are not expected to be involved in arrhythmias in the context of BrS. However, they have also been observed in iPSC-CMs from a BrS patient with a TBX5 gene variant,\(^{17}\) as well as in iPSC-CMs of BrS patients carrying SCN5A mutations.\(^{18}\) In this latter study, those arrhythmic events were linked to abnormal Ca\(^{2+}\) handling, which also led to delayed afterdepolarizations. The investigation of Ca\(^{2+}\) handling in our BrS and Rad R211H ins cell lines showed delayed Ca\(^{2+}\) recapture and abnormal Ca\(^{2+}\) release during recapture in both lines. Combined with the persistent Na\(^+\) current, these results are consistent with the APD prolongation and EAD appearance. This phenomenon could be linked to the immaturity of the cells: on the one hand, a recent study showed that until day 30 of differentiation, iPSC-CMs express a different isoform of NaV1.5 protein.\(^{19}\) On the other hand Ca\(_{\text{v1.2}}\) is not properly localized close to the ryanodine receptor RYR2 due to the absence of T-tubule structures.\(^{20}\) These phenotypic traits seem to be characteristic of BrS in iPSC-CMs but their involvement in triggering arrhythmias in the patient remains uncertain.

The iPSC-CMs of the family members carrying the RRAD p.R211H variant were also characterized by a reduction of the L-type Ca\(^{2+}\) current compared to intra-familial control iPSC-CMs. A reduction of this current has already been involved in some forms of BrS.\(^{5,21}\) Interestingly, RAD has previously been shown to regulate the cardiac L-type Ca\(^{2+}\) current by controlling the Cav1.2 channel trafficking to the sarclemma.\(^{14,22}\) Altogether, these data suggested that the pathophysiological mechanisms of RRAD-related BrS included a combined decrease of both Na\(^+\) and Ca\(^{2+}\) current amplitude, which had never been described before. However, the investigation of the isogenic Rad R211H ins iPSC-CMs only confirmed the loss of function of the Na\(^+\) current. This discrepancy could be explained by the presence of other variant(s) limiting this dual electrical modulation to this particular family. Nonetheless, the isogenic model validates that RRAD p.R211H variant is sufficient to provoke a pro-arrhythmic phenotype and suggests that the main BrS causal defects induced by this variant are a dysregulation of the Na\(^+\) channel and an alteration of the cytoskeleton.

Indeed, in addition to the electrical defects, RRAD p.R211H disorganizes the striated architecture of the cardiomyocyte cytoskeleton, and disturbs the localization of focal adhesions, which leads to cell rounding. Again here, the gain-of-function effect of the mutation RAD GTPase activity is proven, since the overexpression of WT RAD induces similar effects in neonatal mouse cardiomyocytes, and more severely with the overexpression of p.R211H-RAD. RAD is known to interact with the cytoskeleton.\(^{23,24}\) In the present study, RRAD p.R211H acts like a down-regulator of the F-actin cytoskeleton consolidation - most likely through the inhibition of Rho Kinase activity (Supplementary material online, Figure S12), leading to a decrease in cell contractility and focal adhesion formation and maturation.\(^{25}\) These structural defects, which might be limited to the RVOT given the preferred localization of RAD protein in this cardiac region, could lead to decreased cell-to-cell connection and abnormal cardiac conduction,\(^{26}\) and thus play a role in BrS. There are indeed accumulating evidences that structural anomalies in the myocardium, such as fatty infiltrations and fibrosis, may trigger electrical anomalies in relation to BrS.\(^{27–29}\) Furthermore, several susceptibility genes or loci, such as MOG1,\(^{30,31}\) or HEY2, have been shown to regulate not only electrical activity but also cardiac morphogenesis.\(^{32,33}\) Taken together, these data indicate that structural ventricular anomalies may contribute significantly to the expression of the electrical features typical of BrS.

**Study limitations**

One of the limitations of the present study relates to the immature state of the iPSC-CMs. At the electrical level, the lack of \(k_{\text{Ca}}\) and the low \(I_{\text{Na}}\) density prevent the cardiomyocytes to display proper action potential shape and resting membrane potential. At the structural level, the absence of cell polarity and T-tubule structures makes it difficult to study calcium handling and cytoskeleton integrity due to lack of proper localization of the proteins involved. Moreover, Brugada syndrome being related to RVOT, these cells fail to capture the complexity of the phenotype due to lack of cardiac layer specification (endocardium, myocardium, and epicardium) and chamber specification (right or left ventricles/auricles). Despite these limitations, the fact that iPSC-CMs express the main ion currents and display spontaneous contractile activity makes it an accurate model to study the impact of a specific variant at the single cell level in a patient-specific manner.

In conclusion, p.R211H RRAD variant induces a gain of function of RAD, which reduces the amplitude of the Na\(^+\) current, a mechanism already associated with BrS, and induces a persistent Na\(^+\) current. In addition, this variant leads to cytoskeleton anomalies and defects in cell morphology. This impaired structural integrity of cardiomyocytes, which had never been related to BrS, might alter conduction by destabilizing myocardial structure. Furthermore, the fact that the insertion of the mutation in an external control cell line recapitulates the overall phenotype confirms the involvement of the p.R211H RRAD variant in the BrS phenotype observed in the other models. The relative contribution of myocardial structural abnormalities vs. electrical alterations remains uncertain and should be subjected to further investigations.

**Supplementary material**

Supplementary material is available at European Heart Journal online.

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