A Mutation in the G-Protein Gene \textit{GNB2} Causes Familial Sinus Node and Atrioventricular Conduction Dysfunction

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**Rationale:** Familial sinus node and atrioventricular conduction dysfunction is a rare disorder that leads to paroxysmal dizziness, fatigue, and syncope because of a temporarily or permanently reduced heart rate. To date, only a few genes for familial sinus and atrioventricular conduction dysfunction are known, and the majority of cases remain pathogenically unresolved.

**Objective:** We aim to identify the disease gene in a large 3-generation family (n=25) with autosomal dominant sinus node dysfunction (SND) and atrioventricular block (AVB) and to characterize the mutation-related pathomechanisms in familial SND+AVB.

**Methods and Results:** Genome-wide linkage analysis mapped the SND+AVB disease locus to chromosome 7q21.1-q31.1 (2-point logarithm of the odds score: 4.64; β=0); in this region, targeted exome sequencing identified a novel heterozygous mutation (p.Arg52Leu) in the \textit{GNB2} gene that strictly cosegregated with the SND+AVB phenotype. \textit{GNB2} encodes the \( \beta_2 \) subunit (\( G_{\beta_2} \)) of the heterotrimeric G-protein complex that is being released from G-protein–coupled receptors on vagal stimulation. In 2 heterologous expression systems (HEK-293T cells and \textit{Xenopus laevis} oocytes), an enhanced activation of the G-protein–activated K⁺ channel (GIRK; Kir3.1/Kir3.4) was shown when mutant \( G_{\beta_2} \) was coexpressed with \( G_{\gamma_2} \); this was in contrast to coexpression of mutant \( G_{\beta_2} \), with other cardiac ion channels (HCN4, HCN2, and Cav1.2). Molecular dynamics simulations suggested a reduced binding property of mutant \( G_{\beta_2} \) to cardiac GIRK channels when compared with native \( G_{\beta_2} \).

**Conclusions:** A \textit{GNB2} gene mutation is associated with familial SND+AVB and leads to a sustained activation of cardiac GIRK channels, which is likely to hyperpolarize the myocellular membrane potential and thus reduces their spontaneous activity. Our findings describe for the first time a role of a mutant G-protein in the nonsyndromic pacemaker disease because of GIRK channel activation. (Circ Res. 2017;120:e33-e44. DOI: 10.1161/CIRCRESAHA.116.310112.)

**Key Words:** atrial fibrillation ■ bradycardia ■ atrioventricular block ■ dizziness ■ sick sinus syndrome

Sinus node dysfunction (SND) is a slowly progressive disease with a variable natural course that electrocardiographically presents with sinus bradycardia and pauses, sinoatrial exit block, tachycardia–bradycardia syndrome, persistent atrial fibrillation, and sometimes signs of atrioventricular conduction disturbances. Typical symptoms of SND include syncope, dizziness, and palpitations but only rarely cardiac arrest. In most cases, it results from a senescent and age-dependent SND because of intrinsic abnormalities and is, therefore, especially seen in the elderly, mostly accompanied by ischemic heart disease or hypertension.

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E33
In recent years, several studies have discovered causative genes for SND or congenital cardiac conduction disease through candidate gene approaches, linkage analysis, or association studies. To date, these genes are mainly cardiac ion channel genes (eg, for the cardiac pacemaker HCN4, SCN5A, CACNA1D, and TRPM4) or ion channel–associated genes (eg, ANK2), but also structural myocellular proteins (eg, MYH6 and LMNA). Inherited SND was found as a partial and overlapping phenotype in other primary electric heart disorders such as long-QT syndrome, Brugada syndrome, or CPVT (catecholaminergic polymorphic ventricular tachycardia). Moreover, a few HCN4 gene mutations might be associated with atrioventricular block (AVB), atrial fibrillation, or left ventricular noncompaction cardiomyopathy. Recently, SND has been identified as a phenotypic part of autosomal recessively inherited syndromic disorders caused by mutations in SGOL1 gene, encoding a component of the cohesin complex (causing CAID [chronic atrial and intestinal dysrhythmia] syndrome), or in the G-protein–encoding gene GNB5, associated with severe neurocognitive impairment, gastroesophageal reflux, and ophthalmological abnormalities. In Zebrafish morpholino knockdown models of the GNB5 gene, we identified for the first time a mutation in the GNB2 gene encoding the β1 subunit of the heterotrimeric G-protein complex. We provide evidence that the non-synonymous mutation has a downstream effect on the β2-dependent activation of the G-protein–coupled inwardly rectifying potassium channel (GIRK) by increasing the mediated IK,ACH current. The results reveal that GIRK channels have an important role in normal sinus node function in humans and in heart rate control.

Methods

Clinical Evaluation of a Family With SND+AVB
A 3-generation family (25 family members) with an autosomal dominant form of a SND and AVB (sinoatrial disease) was clinically investigated with routine noninvasive cardiac investigations (12-lead ECG, Holter, and, in part, exercise ECGs, thoracic echocardiography, and determination of plasma electrolytes and thyroid function). The diagnosis of idiopathic SND was made in the absence of a structural heart disease and extrinsic causes for SND after thorough examination. Also, the personal and medical history and presence of familial and extracardiac diseases were interrogated. The study was performed in concordance with the revised forms of ethical standards laid down in the Declaration of Helsinki and in accordance with recommendations given by the Ethics Committee of the University of Münster. Written informed consent was obtained from all study participants before inclusion in the study.

Genome-Wide Linkage Analysis and Logarithm of the Odds Score Calculations
Genomic DNA was isolated from leukocytes of EDTA-blood (Invisorb Spin Blood Midi Kit). Initially, 400 highly polymorphic microsatellite markers (LMS-MD-10; Applied Biosystems; average distance: 10 cM) were used for genome-wide linkage analysis. Noninformative markers in particular regions were supplemented with additional, interspersed microsatellite markers that were ascertained from the deCODE marker map (http://www.nature.com/ng/journal/v31/n3/extref/ng917-S13.xls) or the National Center for Biotechnology Information (NCBI) database. DNA fragment lengths were determined after capillary electrophoresis (ABI Prism 3700 Genetic Analyzer; Applied Biosystems) using the GeneScanTM and GenotyperTM 2.0 software modules (Applied Biosystems). Logarithm of the odds (LOD) score calculations were performed using FASTLINK and MLINK version of the LINKAGE 5.1 program package (http://softlib.rice.edu/softlib/fastlink.html). Autosomal dominant inheritance was assumed. Multipoint and 2-point LOD scores were calculated with a disease penetrance of 1.0 because 3
children (ID: 19, 20, and 21) developed the arrhythmia during early childhood. In addition, no genetic heterogeneity and phenocopy within the family was assumed because the incidence of the disease is certainly <1:10000 because of the particular rare phenotype of SND combined with atrioventricular dysfunction. Microsatellite allele frequencies were set to equal (1/n). For calculations, the distances between the microsatellite loci were based on chromosomal maps available from NCBI and deCODE and were transformed into recombination fractions according to Haldane mapping function (MAPFUN version 2.45; http://www.imtech.res.in/pdsb/cata/czm.html).

Targeted Exome Sequencing on Chromosome 7q21.1-q31 and Variant Prioritization

Targeted exome sequencing was commercially done in the index case (ID: 8; Eurofins MWG Operon). The coding region of all genes within the linkage region (chr7:96088156–117301877) was captured using the SureSelect Target Enrichment Kit (Agilent Technologies) followed by sequencing on the Roche FLX454 platform. Raw sequence data were aligned to the human reference genome (hg18) by gsMapper v2.3. Calling and annotation of single-nucleotide variants and short insertions and deletion were performed using the quality-based variant detection tool of the CLC Biogenomic workbench. Only high-quality variants (coverage >15x) that were predicted to lead to potentially serious consequences (stop-gain, stop lost, frameshift, and missense, within 10 bp of a distance to a splice site or coding indel) were considered for further analysis and compared with the publicly available variant databases, that is, dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/), 1000 Genomes database (www.1000genomes.org), and Exome Variant Server (http://evs.gs.washington.edu/EVS/). Variants that were found with a minor allele frequency >0.05% in one of these databases were excluded from further analysis (Figure 2A). The remaining nucleotide variants, including GNB2, were validated by Sanger sequencing with standard procedures (Online Data Supplement).

The potential functional impact of the validated nucleotide variants was calculated by the pathogenicity prediction tools PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), Mutation taster (http://www.mutationtaster.org/), SIFT (http://sift.jcvi.org/), and MutPred (http://mutpred.mutdb.org/). Additionally, the Combined Annotation and Depletion score (http://cadd.gs.washington.edu/), which integrates many diverse annotations into a single measure, was calculated. Validated variants were tested for cosegregation with the disease phenotype by Sanger sequencing in the affected probands 3, 13, and

Figure 1. A, Pedigree of family with autosomal dominant sinus node and atrioventricular conduction dysfunction. ECGs are represented in Online Figure I. Male members are represented by squares and female members by circles. Clinically affected family members are indicated by solid symbols, and open symbols indicate healthy family members. + at the right top of a symbol indicates presence of the GNB2 mutation and – indicates its absence (wild type). Arrow indicates index patient. The assigned alleles for 9 different microsatellite markers are given. In gray, haplotype cosegregating with the familial diseases is marked; the GNB2 gene is located close to marker D7S2480; the identified GNB2 mutation is part of the marked haplotype. B, Ideogram of chromosome 7 with microsatellites markers used in the present study. A 16.8 cM region on chromosome 7q21.1-q31.1 with significant logarithm of the odds (LOD) score encompasses ≈25 Mb genomic sequences with 272 annotated genes. C, Multipoint linkage analysis identified a 16.8 cM region with significant LOD scores (>3.0; y axis) on chromosome 7q21.1-q31.1 including microsatellite markers D7S491 and D7S2460 (x axis). KcM indicates Kosambi centiMorgan.
Variant with a coverage >15x (after TES) 739 variants in 149 genes

Variant with potentially serious consequence (i.e. stop-gain, stop-loss, frameshift, missense, within 10 bp of a distance to a splice site or coding indel) 77 variants in 34 genes

Variant absent or very rare (MAF<0.05%) in EVS, dbSNP, 1000 Genomes 7 variants in 7 genes

Validated variant cosegregating with the disease in the family
GNB2, c.155G>T

Figure 2. A. Results from targeted exome sequencing (TES) of genes on chromosome 7q21.1-q31.1 and prioritization strategy applied to nucleotide variant (family member B). bp indicates base pairs; EVS, Exome Variant Server; dbSNP, Single-Nucleotide Polymorphism Database; and MAF, minor allele frequency. B. Electropherogram of exon 4 of the GNB2 gene on chromosome 7q22.1 showing a heterozygous transversion c.155G>T predicted to result in a nonsynonymous amino acid exchange (p.Arg52Leu). C. Multiple orthologous sequence alignment of human Gβ2 protein region containing the R52L mutation. Identical amino acids are indicated by an asterisk in the lower lane. The mutated amino acid residue is indicated in bold. D. Multiple paralogous sequence alignment of human Gβ, amino acid sequences. E. mRNA expression of GNB2 in different heart compartments and brain normalized to GAPDH. A indicates atrial; AV, atrioventricular node; b, brain; PF, Purkinje fiber; SN, sinus node; and V, left ventricle. The 2−ΔΔCt method was used to describe the relative mRNA expression. To compare the expression of a target gene in a given tissue with the expression in atrium, the 2−ΔΔCt method was used.

Transcriptional Profiling in Human Cardiac Tissue

Total RNA from human atrium, ventricle, sinus node, atrioventricular node, and Purkinje fibers was purchased from Analytic Biological Services Inc. The company states that the tissue samples from atrium, ventricle, sinus node, and Purkinje fibers were collected 4 days postmortem from a 41-year-old white woman who died because of liver failure. In addition, atrioventricular node samples were obtained from a 61-year-old white man who experienced asthma and COPD (chronic obstructive pulmonary disease). For both donors, no cardiovascular, kidney/urinary, neurological, or digestive disorders were reported by the company. ABS Inc states that their postmortem collections are accredited by the government and that they got the donor’s consent before removing any tissues for research. Total RNA obtained from human brain was purchased from Zyagen. The company states that the tissue samples from atrioventricular node; b, brain; PF, Purkinje fiber; SN, sinus node; and V, left ventricle. The 2−ΔΔCt method was used to describe the relative mRNA expression. To compare the expression of a target gene in a given tissue with the expression in atrium, the 2−ΔΔCt method was used.

Cloning and Site-Directed Mutagenesis

cDNA clones of human GNB2 (NM_005273.3), GNB2-Flag (NM_005273.3), GNG2 (NM_053064.4), GNG2-HA (NM_053064.4), and GNAI2 (NM_002239) were ordered from Missouri S&T cDNA Resource Center. The GNB2 mutation c.155G>T was introduced by using the Quik-Change site-directed mutagenesis kit (Stratagene).

For electrophysiological experiments, GNB2 and GNG2 each were subcloned into pRES2-AcGFP1 (Clontech) or pRES2-dsRED-Express2 (Clontech). For subcloning of the cDNAs encoding the relevant GIRK channel subunits, the full-length KCNJ3 (NM_002239; vector: pCMV6-XL5 (SC118769)) and KCNJ5 (NM_000890.3; vector: pCR4-TOPO (IRATp970C11115D)) were obtained from OriGene or Source BioScience. For simultaneous heterologous expression of KCNJ5 and KCNJ3, a bidirectional mammalian expression vector (pH1-CMV1, Clontech) was used.

Gβ2-Gγ2 and Gβ2-Gγ2 Interaction Assay

To study the interaction of native or mutant Gβ2 with the –Gγ2 subunit and the Gγ2 subunit, coprecipitation analyses of in vitro expressed Gβ2-Flag, Gγ2-Flag, and Gα subunits were performed. Therefore, human embryonic kidney (HEK-293T) cells were transfected with native pcDNA3.1-GNB2-Flag or pcDNA3.1-GNB2-Flag and in addition with pcDNA3.1-GNG2-HA and pcDNA3.1-GNAI2 (1 µg each) using 4 µL x-tremeGENE 9 Transfection Reagent (Roche) in 100 µL Opti-MEM per dish. Whole-cell lysates were obtained 48 hours after transfection (Online Data Supplement) and immunoprecipitated with anti-HA agarose using the Pierce HA Tag IP/Co-IP Kit (Thermo Scientific). Western blot analysis of coimmunoprecipitated Gβ2 and Gγ2 subunits, as well as Gγ2, was performed as described in the Online Data.
Electrophysiological Studies

HEK-293T cells were transfected with 1 μg pBI-CMV-KCNJ3/ KCNJ5 in combination with 1 μg pIREs2-gfp-GNB2 or pIREs2- acGFP1-GNB2. Transfection Reagent (Roche) in 100 μL Opti-MEM per dish. One day after transfection, the cells were plated on new 30-mm dishes at a density of 50,000 cells per dish. Whole-cell recordings were performed at room temperature (20–22°C) 2 days after transfection using an EPC-10 amplifier (HEKA Electronics Inc.). Currents were digitized with a sampling rate of 10 kHz and filtered at 3 kHz. Pipettes were pulled from borosilicate glass to resistance 3 to 5 MΩ and filled with an internal solution containing 120 mmol/L KCH3O3S, 4 mmol/L NaCl, 1 mmol/L MgCl2, 0.5 mmol/L CaCl2, 10 mmol/L HEPES, 10 mmol/L EGTA, 3 mmol/L MgATP, 0.3 mmol/L GTP-Tris, pH 7.2. External solution contained 137 mmol/L NaCl, 6 mmol/L KCl, 10 mmol/L HEPES, 2 mmol/L CaCl2, 2 mmol/L MgCl2, and 10 mmol/L glucose, pH 7.3.

The pipette potential was set to zero before seal formation, and capacitive transients were compensated using C-fast for pipette capacity correction and, subsequently, C-slow for cell-capacity compensation (PatchMaster; HEKA Electronics). Membrane voltages were corrected for a 10 mV liquid junction potential. Cells were voltage-clamped for 1000 ms at 0 mV, and channel currents were elicited with 2000-ms pulses to potentials from −140 mV to +60 mV in +20 mV increments (cycle length 3000 ms). In addition, current activation kinetics was analyzed fitting a single exponential function to derive the activation time constant (Tactivation; software by M. Pusch, http://users.ge.ibf.cnrt.at/pusch/programs-mik.htm). The analysis was conducted for the current traces that showed clear activation kinetics (~140, −120, and −100 mV). The other current traces were not evaluated because of the small activating components and relatively high capacitance artifacts.

Molecular Dynamics Modeling

Human Kir3.1 and Kir3.4 were used to construct consensus models using the software YASARA Structure 10 (YASARA Biosciences GmbH, Vienna, Austria). GIRK subunits were modeled individually using 4KF.pdb as main template. The heteromeric GIRK channel complex was modeled by positioning Kir3.1 next to Kir3.4 next to Kir3.4 and to Kir3.4. Thus, the respective subunit Kir3.1 is neighbored by Kir3.4. Native or mutant Gβ and several ligands (8 PIP2 molecules, Na+ and K+) were positioned similar as in 4KF.pdb. The modeled GIRK-Gβ complex was energy-minimized using YASARA structure 10 as described before.17 Finally, the model complex was incorporated into a lipid membrane, and 2 ns all-atoms mobile molecular dynamics simulations using force field AMBER03 (2 fs time steps) simulation was performed as previously described.17

Results

Clinical Phenotype of a Family With Autosomal Dominant Sinoatrial Disease

We investigated a German 3-generation family with an autosomal dominant form of sinoatrial disease. The disease phenotype includes sinus bradycardia as one of the first electrocardiographic signs and later development of sinoatrial disease, together with atrioventricular conduction disturbance (AVB) of lower to higher degree (including AVB III°/atrioventricular dissociation) and atrial fibrillation. Review of available ECG data (Table; Online Figure I) revealed at least 11 affected family members with SND, SND+AVB, or atrial fibrillation. Fourteen family members were clinically unaffected (not shown).

To date, 6 patients received a dual-chamber pacemaker, ranging from age 8 years to the third or fourth decade of first implantation (Table). In the surface ECGs, QRS duration, P-wave width, and QT interval were normal in all affected individuals; also, there were no clinical signs for Brugada syndrome at baseline. The family history was completely unremarkable for sudden cardiac death. Available echocardiographic data showed no structural heart disease, for example, abnormal ventricle dimension or function or anatomic defects, and there were no extracardiac disease signs. In addition, there were no extrinsic causes for SND. Clinical symptoms related to SND+AVB were syncope in 3 GNB2 mutation carriers, the majority achieved pacemaker implantation because of bradycardia rather than heart rate increase during exercise.

Genetic testing of affected family member 8 and 13 for the known genes involved in sinus node or atrioventricular node dysfunction (HCN4, SCN5A, LMNA, CACNA1D, and TRPM4) was previously performed without identification of an indicative mutation.

Genome-Wide Linkage Analysis to Identify the Disease Locus

For genome-wide linkage analysis, 23 consenting members of the family (11 affected and 12 unaffected; Figure 1A) were genotyped by using a linkage panel encompassing 400 highly polymorphic microsatellite markers. Parametric 2-point analysis revealed only one chromosomal region with significant linkage (ie, LOD score >3) on chromosome 7q (LOD score = 4.34). This candidate region was further investigated by 16 additional microsatellite markers with a final average intermarker distance of 1.62 cm. Significant linkage was detected for the chromosomal interval between markers D7S491 and D7S2502 (Figure 1B) with a maximum LOD score of 4.64 at D7S2480 and D7S471 (Online Table II; recombination fraction Θ = 0).

Multipoint linkage analysis further demonstrated at least 7 microsatellite markers within the chromosome 7 region with positive LOD scores (Z maximum = 4.6) that were robust for different recombination fractions (Figure 1C). Within this region (chromosome 7q21.1-q31.1), haplotypes were constructed in family 10018, and all affected family members were found to share a common haplotype in linkage disequilibrium spanning ≈16.8 cm. This haplotype block was also absent in all unaffected family members (Figure 1A). According to the NCBI Map Viewer database (Build 37.3), the refined disease locus (chr7:96088156–117301877) contained 272 positional candidate genes, of whom, however, none had been previously associated with an inherited form of an arrhythmia or cardiomyopathy.

Target-Specific Exome Sequencing Genes on Chromosome 7q21.1-q31.1

In the index patient (8), we performed custom-targeted exome sequencing of all 7q21.1-q31.1 genes. 79.1% of total reads achieved mapped to the target interval on chromosome 7. Coverage of the captured regions was 96% and 90.6% for at least 10x depth of coverage. The mean sequencing depth was 38x per base. A total of 739 nucleotide variants (in 149 genes) with a coverage of at least 15x were identified. Among them, 77 nucleotide variants in 34 different genes had a predicted functional consequence (ie, stop-gain, frameshift, and missense, within 10 bp of a distance to...
Table. Clinical Features of Patients With Congenital Sinus Node and Atrioventricular Conduction Dysfunction (SND+AVB) and a Heterozygous GNB2 Gene Mutation (c.155G>T, p.Arg52Leu)

<table>
<thead>
<tr>
<th>PID</th>
<th>Current Age, Sex</th>
<th>Clinical Symptoms</th>
<th>Pacemaker Implantation (Age)</th>
<th>ECG Features</th>
<th>Normal ECG Intervals (During SR)</th>
<th>Structural Cardiac/ Extracardiac Abnormalities</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Died, M</td>
<td>NA</td>
<td>55 y</td>
<td>Sinus bradycardia</td>
<td>45/min QRS, P-wave, QTc</td>
<td>None (TTE)/none</td>
</tr>
<tr>
<td>8</td>
<td>53 y, F</td>
<td>Syncope (46 y)</td>
<td>46 y</td>
<td>35 y: binodal disease, sinoatrial block (SAB+AVB II° with long conducting interval; Online Figure IA) 49 y: baseline ECG with continuous AV pacing, 60/min; exercise ECG with intrinsic SR, AVB, HR_{min} 173/min 51 y and 53 y: Baseline ECG with continuous ventricular pacing, intermittent AV pacing. Postpacing VPB (Online Figure IB)</td>
<td>46–54/min QRS, P-wave, QTc</td>
<td>None (TTE)/None</td>
</tr>
<tr>
<td>11</td>
<td>62 y, F</td>
<td>Asymptomatic</td>
<td>No PM</td>
<td>33 y: Intermittent binodal disease, SAB+AVB 37 y: sinus bradycardia; exercise ECG with SR, HR_{min} 150/min</td>
<td>52/min QRS, P-wave, QTc</td>
<td>None (TTE)/none</td>
</tr>
<tr>
<td>13</td>
<td>66 y, F</td>
<td>Chronotropic incompetence</td>
<td>48 y+61 y</td>
<td>47 y: AVB II° Wenckebach, intermittent AFIB (Online Figure IC)</td>
<td>54/min QRS, P-wave, QTc</td>
<td>None (TTE)/none</td>
</tr>
<tr>
<td>15</td>
<td>64 y, F</td>
<td>Syncope</td>
<td>44 y+51 y</td>
<td>44 y: sinus bradycardia, AFIB (tachycardia–bradycardia) 62 y: AFIB 63 y: AFIB+ventricular pacing, VPB</td>
<td>81/min* 68/min* QRS, QTc</td>
<td>None (TTE)/none</td>
</tr>
<tr>
<td>19</td>
<td>27 y, F</td>
<td>Asymptomatic</td>
<td>No PM</td>
<td>17–20 y: dominant AVB II°, intermittent AVB III°, also intermittent SBr+AVB I° (260 ms). 23 y: SBr+AVB I° (260 ms)</td>
<td>26–123/min 51/min QRS, P-wave, QTc</td>
<td>None (TTE)/none</td>
</tr>
<tr>
<td>20</td>
<td>22 y, M</td>
<td>Chronotropic incompetence</td>
<td>8 y+14 y</td>
<td>2 y: sinoatrial disease, intermittent AVB II°–III° 14 y–16 y: baseline ECG with continuous AV pacing 21 y: exercise ECG reached 81% of expected HR_{min} without stimulation; at baseline constant AV pacing (Online Figure ID)</td>
<td>62/min 63–166/min QRS, P-wave, QTc</td>
<td>None (TTE)/none</td>
</tr>
<tr>
<td>21</td>
<td>25 y, M</td>
<td>Syncope (18 y)</td>
<td>19 y</td>
<td>15 y: sinoatrial disease; AVB III°, intermittent AFIB. During flecainide therapy increasing need for pacing, but broad complex tachycardia, PVS without inducibility for supra- or ventricular tachycardia 20 y: SBr, intermittent AFIB 20 y–25 y: baseline ECG with continuous AV pacing 22 y: exercise ECG reached 79% of expected HR_{min} without stimulation; at baseline constant AV pacing</td>
<td>45/min 63–158/min QRS, P-wave, QTc</td>
<td>None (TTE)/none</td>
</tr>
<tr>
<td>34</td>
<td>40 y, M</td>
<td>Asymptomatic</td>
<td>No PM</td>
<td>SAB, AVB</td>
<td>45–65/min QRS, P-wave, QTc</td>
<td>NA</td>
</tr>
<tr>
<td>22</td>
<td>36 y, M</td>
<td>Asymptomatic</td>
<td>No PM</td>
<td>18 y: sinus bradycardia (Online Figure IE) 19 y: complete AVB at rest; during exercise 1:1 AV conduction and chronotropic response</td>
<td>42/min 45–170/min QRS, P-wave, QTc</td>
<td>None (TTE)/none</td>
</tr>
<tr>
<td>37</td>
<td>28 y, M</td>
<td>Asymptomatic</td>
<td>No PM</td>
<td>14 y: sinus bradycardia</td>
<td>46–50/min QRS, P-wave, QTc</td>
<td>None (TTE)/none</td>
</tr>
</tbody>
</table>

Representative ECG examples are shown in Online Figure I. AFIB indicates atrial fibrillation; AV, atrioventricular; AVB, atrioventricular block; F, female; HR, heart rate; M, male; NA, not available; PID, patient identifier; PM, pacemaker implantation; PVS, programmed ventricular stimulation; SAB, sinoatrial block; SR, sinus rhythm; and VPB, ventricular premature beats.

*During AFIB, HR, AVB, SR, AV, VPB, SAB, PM, and PVS.
a splice site or coding indel) and were taken into account for further analysis. These nucleotide variants were further narrowed down to finally 7 heterozygous variants that were also absent or rare (minor allele frequency <0.05%) in population control databases such as dbSNP, the Exome Variant Server, and the 1000 Genomes database (Figure 2A). Three of the 7 variants could be independently confirmed in index patient (ID: 8) by Sanger sequencing (Online Table III) and were further tested for cosegregation with the clinical phenotype of SND+AVB in family members. Finally, only the heterozygous nucleotide transversion in the GNB2 gene (c.155G>T; NM_005273.3; Figure 2B) encoding guanine-nucleotide-binding protein (G-protein) subunit β-2 (Gβ2) was identified by Sanger sequencing to be present in all affected family members, and vice versa was absent in all unaffected family members (Figure 1A) and in population control databases with >10,000 individuals (Exome Variant server; http://evs.gs.washington.edu/ EVS/) and the Exome Aggregation Consortium (exac.broadinstitute.org/) databases. This nucleotide variant leads to a substitution of the basic amino acid arginine to the neutral amino acid leucine at position 52 of the GNB2 protein sequence (p.Arg52Leu; P62879). The affected amino acid is located just before the first WD repeat motif of the amino acid sequence and reveals a high degree of paralogous and orthologous conservation (Figure 2C and 2D). PolyPhen-2, MutPred, SIFT, and Mutation taster concordantly predicted this variant to be deleterious to Gβ2 function, and the calculated Combined Annotation and Depletion score of 24.8 predicted this variant to be among the 1% most deleterious substitutions in the human genome.

We further investigated the complete 9 coding exons and exon–intron boundaries of GNB2 in other unrelated patients with diagnosis of idiopathic or familial bradycardias, that is, sinus bradycardia or SND (n=50) and AVB (n=18). Among this population, no further GNB2 mutation was identified. However, none of these patients revealed such a particular ECG phenotype of a sinoatrial disease (SND+AVB) as it has been seen in GNB2 R52L mutation carriers.

**Tissue Expression Profiling of GNB2 in the Human Heart Compartments**

We investigated the expression of the GNB2 gene in different human heart tissues and human brain by quantitative reverse transcription-polymerase chain reaction. We first characterized available, obtained RNA samples from different heart compartments for transcripational detection of NPPA (ANP), a hormone preferentially expressed by atrial tissue; TBX3, a transcription factor expressed in the conduction system; GJA5 (Cx40), a gap junction protein (expressed preferentially in the conduction system, absent in ventricle); and HCN4, the cardiac pacemaker channel gene, preferentially expressed in the sinus node. NPPA revealed highest expression in a RNA sample obtained from atrial tissue (Online Figure IIA), whereas TBX3 and GJA5 were predominately expressed in the sinus and atrioventricular nodes. As known, highest expression of HCN4 was seen in the sinus node. The observed low expression of NPPA, but higher expression of TBX3, GJA5, and HCN4 in samples from the conduction system when compared with the ventricle suggested only little contamination with ventricular cells during sample preparations (Online Figure IIA).

Expression of the GNB2 gene was shown in all human cardiac subcompartments and in the human brain. Compared with the expression level in the atrium, highest transcriptional levels were observed in the human atrioventricular node and brain (Figure 2E).

Because heterotrimeric G-proteins are composed of several α, β, and γ subunits (in humans: 5 Gβ subunits and 12 Gγ subunits are known), we also compared the relative cardiac expression of GNB2 with the other 4 human GNB genes and 3 other GNG genes, all known to be expressed in cardiomyocytes.18 We confirmed gene expression of all 5 human GNB genes (ie, GNB1-5) and the 3 analyzed GNG genes (GNG2, GNG7, and GNG12, respectively) in human heart compartments and in the brain. The overall highest relative expression level in the heart was seen for GNB1 and GNG12, followed by GNB2, GNB4, GNG2, and GNG7. GNB3 and GNB5 revealed lowest relative expression values (Online Figure IIIB). Because other Gβ subunits were shown to be expressed in the cardiac conduction tissue, they also may have a physiological role in cardiac impulse generation and conduction.

**Influence of Gβ2Arg52Leu Mutant Protein on G-Protein Complex Formation**

The heterotrimeric G-proteins are composed of α, β, and γ subunits and are important for cellular signal transduction. Gγ and Gβ subunits form a stable dimer that is, in the inactive state, associated to Goα bound to GDP. Activated by a ligand, GDP is exchanged by GTP, which results in the dissociation of the Goα from GpGγ.19

Because the biological function of the G-protein complex depends on the interaction of the 3 G-protein subunits, we first investigated whether the mutant Gβ2 subunit (Gβ2Arg52Leu) has an altered protein stability and binding affinity to the Gγ subunit or the Goα subunit compared with native Gβ2.

In protein stability assays, the degradation rates of Gβ2Arg52Leu did not differ from those of native Gβ2 protein (Online Figure IIIA). In addition, coimmunoprecipitation assays showed no altered (ie, reduced or enhanced) binding properties to Goα and Gγ subunits (Figure 3). In immunofluorescence and Western blot analysis of membrane protein fractions deriving from HEK-293T cells (transfected with mutant Gβ2Arg52Leu and Gγ), the GpGγ complex revealed a similar subcellular localization at the cell membrane as seen in HEK-293T cells transfected with native Gβ2 and Gγ (Online Figure IIIB).

**Effect of Mutant Gβ2 on GIRK Channel Activity: A Gain of Function**

After activation, the GTP-bound Goα subunit dissociates, and the released heteromeric GpGγ dimer transduces signals to several downstream effector proteins. In the human heart, 3 different ion channels are involved in heart rate and atrioventricular conduction regulation and are directly modulated by GpGγ: the GIRK that mediates the acetylcholine-dependent potassium current (IacKACH), direct activation by GpGγ dimers (18) and 2 Ca2+ channel, the L-type Ca2+ channel (LTCC) Cav1.2 (21) and the T-type Ca2+ channel (TTCC) Cav3.2,22 which are both inhibited by GpGγ protein complex. In the human heart, the expression of the main subunit (CACNA1H) of the Cav3.2 channel is mostly restricted to embryonic tissue,23 making a potential role in SND+AVB unlikely.
Thus, we first focused on GIRK channels (mediating I_{K,ACCh}) which are heterotetramers of 2 channel subunits: GIRK1 and GIRK4 (also known as Kir3.1 and Kir3.4). GIRK channels are involved in the negative chronotropic effect of the parasympathetic nervous system as a downstream effector of G_{i}βγ. To investigate the effect of mutant Gβ2(Arg52Leu) in the presence of Gγ2 on GIRK channel activity, we performed whole-cell patch-clamp analyses on HEK-293T cells cotransfected with Kir3.1+Kir3.4. GIRK currents (Figure 4A through 4C) revealed a significant increase of the inward and outward current amplitudes. The results of the measurements showed in both solutions at 35 mV, the Kir3.1/Kir3.4+Gβ2 complex did not introduce an unexpected or unusual current modulation to HCN2 (Online Figure VE and VF) or to HCN4 (I_f) modulation. However, the Gβ2-Arg52Leu in the presence of Gγ2 activated GIRK currents (Online Figure VA). Coexpression of this Cav1.2 channel complex with the Gβ2γ2 complex reduced the Cav1.2 current amplitudes, as previously described (Online Figure VA through VC). Strikingly, the Cav1.2 current reduction was similar for wild-type Gβ2γ2 and Gβ2-Arg52Leuγ2 (Online Figure VA through VC). The peak current of the bell-shaped current–voltage relationship was also shifted to a similar extent for native and mutant Gβ2-containing Cav1.2 channel complexes (Online Figure VB). This previously described rightward shift of the voltage dependence of n=10 mV was also present when analyzing only oocytes with similar amplitudes of <5 pA (Online Figure VF), excluding that this difference is caused by larger current amplitudes of native Cav1.2 channels.

Next, we coexpressed HCN pacemaker channels, which are not modulated by Gβγ proteins with native or mutant Gβ2γ2 to exclude that the R52L mutation causes an aberrant HCN4 or HCN2 (I_L) modulation. However, the Gβ2-Arg52Leuγ2 complex did not introduce an unexpected or unusual current modulation to HCN2 (Online Figure VE and VF) or to HCN4 channels (Online Figure VG and VH).

Taken together, coexpression of mutant GNB2 as part of the Gβ2-Arg52Leuγ2 complex does not result in an aberrant Cav1.2, HCN2, or HCN4 channel modulation, which further supports that the enhanced I_{K,ACCh}-mediated channel activation is the most likely substrate associated with SND+A VB.

Molecular Modeling
A GIRK (Kir3.1/Kir3.4/Kir3.1/Kir3.4) model including ligands (4 K+ in the selectivity filter, 8 PIP2 in membrane domain, and 4 Na+ in Na+ binding sites) was generated, and 4 Gβ2 were docked. The whole complex was incorporated into a lipid membrane in a water-filled box (0.9% NaCl), and a short
molecular dynamics simulation was conducted. Residues Arg52 and Leu52 are positioned at the Gβ2–GIRK interface (Figure 5). Analyses of the model after molecular dynamics simulation revealed that Arg52 contacts 3.24 residues with about 4.54 contacts per contacted residue. On the contrary, residue Leu52 contacts only 1.75 residues with only 4.00 contacts per contacted residue, indicating largely reduced interaction (Online Table IV). These data indicate that mutant Gβ2-Arg52Leu reduces the steric interaction at the GIRK–Gβ2 interface.

Discussion

Using a strategy entailing a genome-wide linkage analysis and targeted exome sequencing of the candidate chromosomal region, we identified a nonsynonymous mutation in the G-protein gene \textit{GNB2} on chromosome 7q21.1-q31.1 that encodes one of the 5 human β subunits as part of heterotrimeric G-protein complexes. This is the first disease gene for an autosomal dominant form of SND combined with AVB and atrial fibrillation with an isolated cardiac phenotype and direct involvement of a GIRK channel regulator. Heterotrimeric G-protein complexes are composed of Go, Gβ, and Gγ subunits. On activation through G-protein–coupled receptors, Gα dissociates from the Gβγ complex and each component acts downstream effectors in diverse physiological processes.19 In the human heart, Gβγ is a direct activator of the GIRK, 20 which is highly expressed in the sinoatrial node and significantly modulates the negative chronotropic effects under vagal stimulation. In transgenic mouse models, a reduction of functional Gβγ produces disturbances of spontaneous depolarization within the sinus node.24 Therefore, we analyzed whether the downstream activation of GIRK channels might be affected by presence of the mutant G-protein subunit (Gβ2-Arg52Leu). In 2 different heterologous expression systems, we concordantly observed a significant reduction of the inward rectification of the GIRK channel and, thus, a significant increase of IK,ACh currents in the physiologically relevant range after activation by mutant Gβ2-Arg52Leu complex. This effect of the mutant Gβ2γ subunit seems to specific for GIRK channels because no significant differences in modulation of Cav1.2, HCN4, and

Figure 4. Electrophysiological characterization of Kir3.1/3.4 currents in presence of native Gβ2γ and mutant Gβ2-Arg52Leuγ as measured in whole-cell patch-clamp configuration in HEK-293T cells. A, Representative basal Kir3.1/3.4 current traces in HEK-293T elicited by the pulse protocol in absence (upper tracings) and presence (middle and lower tracings) of G-proteins. Arrows indicate the zero current level. B, Current–voltage relationship of Kir3.1/3.4 channels measured using the illustrated pulse protocol. C, Current densities of Kir3.1/3.4 channels at a potential of −60 and +20 mV in presence of native Gβ2γ and mutant Gβ2-Arg52Leuγ. D, Activation time constants of Kir3.1/Kir3.4 channels (Tactivation) derived from single exponential fits of the Gβ2γ-evoked Kir3.1/Kir3.4 current activation time course at 3 different voltage pulses (−140 mV, −120 mV, −100 mV, respectively). Data are provided as means±SEM. P values calculated in ANOVA refer to the native Kir3.1/3.4 Gβ2γ. *P≤0.05; **P≤0.01; ***P≤0.001.
HCN2 channels and their mediated currents ($I_{\text{Ca,L}}$, $I_f$) have been observed when native and mutant $\beta_2\gamma_2$ complexes were compared. We, therefore, assume that the $\beta_2$ mutation preferentially leads to an enhanced activation of the cardiac GIRK channels, which then cause a hyperpolarization of the membrane potential in the sinoatrial (and atrioventricular) cells and, in consequence, a slowed impulse generation and conduction. As a limitation, we have not addressed potential effect of mutant $\beta_2\gamma_2$ complexes on the T-type calcium channels (Cav3.1 and Cav3.2) because Cav3.1 has been shown not to be modulated by $\beta_2\gamma_2$ and expression of Cav3.2 is only high in the embryonic sinus node, and we have also not analyzed the effect on the Cav1.3 channel, which is associated with congenital SND5 but not known to be regulated by G$\beta\gamma$.

We demonstrated expression of $\beta_2$ in the human sinus node and atrioventricular node. However, also the other G$\beta$ subunits are expressed in the heart conduction system, and additionally, the 5 human G$\beta$ proteins are highly similar that leads to the question whether the other G$\beta$ subunits may also be involved in heart rate regulation. Interestingly, recently, nonsense mutations in $\beta_5$ have been linked to a syndromic disorder, which is, in addition to neuronal and ophthalmic phenotypes, also clinically characterized by sinus bradycardia. It was demonstrated that G$\beta_5$ is crucial for parasympathetic control of heart rate. However, as G$\beta_5$ is the only G$\beta$ subunit that has been demonstrated to fail to activate GIRK, a pathomechanism different from the one described for the $\beta_2$ mutation can be expected. In addition, a bradycardic phenotype has been described for $\beta_5$ null mice. But it was assumed that $\beta_3$ is not directly involved in the G-protein–coupled receptor signaling activity that controls heart pacemaker activity as hearts isolated from these mice responded equivalently to muscarinic receptor- and $\beta$-adrenergic receptor stimulation. In humans, homozygous or compound heterozygous mutations in $\beta_3$ have been described in inherited retinal diseases, but no cardiac phenotype has been demonstrated for these patients to date.

The G-protein $\beta$ subunit forms a propeller-like structure containing 7 blades. Each blade is made by a $\beta$-sheet of 4 anti-parallel $\beta$ strands. The mutant residue Arg52 of G$\beta_5$ is located within such a $\beta$-sheet element forming blade 7 on one edge of the $\beta$ propeller. This region is not located within the parts of the protein that were shown to be essential for the binding to G-protein $\gamma$ or $\alpha$ subunits, and in line with this, we could not observe an altered binding of G$\beta_2\gamma_2$ to G$\alpha_\gamma$ and G$\gamma_\gamma$ subunits. However, from the crystal structure of the mammalian GIRK2-$\beta_2\gamma_2$, G-protein complex, the residue Arg52 was identified as part of the GIRK binding interface, which was also seen in the molecular model of GIRK1/4 and G$\beta_2\gamma_2$. Interestingly, analyses of the model after molecular dynamics simulation indicated that mutant G$\beta_2\gamma_2$ indeed modifies the interaction at the GIRK–G$\beta_2\gamma_2$ interface. The observed effect on GIRK activation is, therefore, probably because of an altered (reduced) binding of the G$\beta_2\gamma_2$ complex to GIRK. To identify amino acid residues in G$\beta_2\gamma_2$ that are essential for GIRK activation, Mirshahi et al replaced distinct amino acid residues in the G$\beta_2$ subunit to the corresponding residue in G$\beta_5$ and tested the ability of the mutant G$\beta_5$ subunits to activate GIRK. Of note, it was demonstrated that the expression of G$\beta_1\gamma_2\gamma_2$, slightly enhances the GIRK channel current, which additionally underlines a functional importance of residue Arg52 within the G$\beta_2\gamma_2$ complex.

In addition to the reports on $\beta_5$ and $\beta_3$, variants in $\beta_1$ and $\beta_4$ have been reported in human disorders distinct from cardiac arrhythmias. Two mutations were identified in the $\beta_4$ gene in patients with intermediate Charcot–Marie–Tooth neuropathy. Interestingly, one of the reported $\beta_4$ mutations (p.Gly53Asp) is located in close proximity to the residue Arg52 in G$\beta_2$, and it was demonstrated that this variant causes a defect in the G-protein–coupled receptor signaling cascade and impairs the activation of PLC$\beta_5$. Recently, germline de novo mutations in $\beta_1$ have been linked to severe neurodevelopmental disability.

Expression of G$\beta_2$ is not limited to nodal tissue and was also confirmed in brain. However, in contrast to the autosomal dominant G$\beta_2$ gene mutation with an isolated cardiac phenotype, the identified mutations in the $\beta_2$ gene lead to a syndromic (mostly extracardiac) phenotype if both parental alleles were mutant. Because $\beta_5$ is expressed in various tissues, the absence of functional G$\beta_5$ may lead to many different cell-specific dysfunctions resulting in a complex clinical phenotype. In contrast, the heterozygous mutation in G$\beta_2$ increases the activation of an ion channel with a dominantly
cardiac-specific function, and this might explain the isolated cardiac phenotype in a heterozygous setting.

Only a few, single mutations have been reported in the Kir3.4 subunit of the cardiac GIRK channel. The reported mutations were mainly identified in patients with long-QT syndrome and were primarily associated with a loss-of-channel function and decreased IK,ACh currents. However, GIRK4 knockout mice show a delayed recovery of resting heart rate after β-adrenergic stimulation, which underlines the role of GIRK channels in sinus node physiology and heart rate regulation. In this line, recent work on transgenic mice with SND either because of ablation of CACNAID (encoding the α subunit of the L-type Cav1.3 calcium channel) or HCN4 (underlying the hyperpolarization-activated I current) showed that manipulation of GIRK is preventive of SND in these mice models and thereby emphasize cardiac GIRK channels as a pharmacological target for inhibition and thereby improvement of SND.

We have identified a mutation in a G-protein gene (GNB2) for a nonsyndromic, isolated SND+AVB and, moreover, demonstrated that the mutant Gβ2 protein is associated with a significant increase in Gβ2γ complex–mediated GIRK channel activation. This is likely to be a plausible explanation for sinus and atrioventricular nodal arrhythmias in the mutation carriers and underlines the importance of GIRK channels in human sinus node physiology.

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Disclosures

None.

References


A Mutation in the G-Protein Gene GNB2 Causes Familial Sinus Node and Atrioventricular Conduction Dysfunction

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Supplemental Material

Detailed Methods

Sanger sequencing
Sanger sequencing was used (1) for secondary validation of the gene variants identified after targeted exome sequencing of chromosome 7q21.1-q31.1, (2) to analyse family members of family 10018 and other patients for presence of GNB2 variants and (3) for genetic analyses of two affected family member (10018-8 and 10018-13) for known genes involved in congenital sinus node and atrioventricular conduction dysfunction (HCN4, SCN5A, LMNA, CACNA1D, TRPM4). PCR primers for amplification and DNA sequencing of all coding exons and exon-intron boundaries of the analysed genes were designed using the NCBI Primer-BLAST tool. For variant validation PCR primers were designed so that they contained the mutation sites and their flanking regions. PCR products were enzyme purified with ExoSap-IT (USB) and sequenced in both directions with BigDye Terminator Ready reaction mix on a 3500xL Genetic Analyzer (Applied Biosystems). Obtained sequences were compared with the reference sequences (GNB2: NM_005273.3, HCN4: NM_005477.2, SCN5A: NM_198056.2, LMNA: NM_170707.2, CACNA1D: NM_000720.1 and NM_001128840.2, TRPM4: NM_017636.3).

Cell culture
Human embryonal kidney HEK-293T cells (ACC 635, DSMZ) and African green monkey kidney cells COS-7 (ACC 60, DSMZ) were cultivated in Dulbecco’s modified eagle medium (DMEM, Sigma) supplemented with 2 mmol/L L-Glutamine, 100 U/10 µg/ml Penicillin/Streptomycin and 10% FCS (PAA Laboratories) at 37 °C and 5% CO₂. Transfections were performed 24 h after seeding HEK-293T cells on 30 mm dishes.

Immunocytochemistry and confocal microscopy
For immunofluorescence analysis, transfections were performed 24 h after seeding COS-7 on Poly-L-Lysin coated 8-well glass CultureSlides (BD Falcon). For heterologous expression pcDNA3.1-GNB2-Flag or GNB2Arg52Leu-Flag and pcDNA3.1-GNG2-HA was transfected with x-tremeGENE 9 Transfection Reagent (Roche) according to manufacturer’s instructions. In immunofluorescence studies, COS-7 cells were co-transfected with pDsRed-Monomer-F Hyg (Plasmamembrane, Clontech) to investigate the subcellular localization of wild type and mutant Gβ2. After the incubation period of 48 h after transfection COS-7 cells on 8-well CultureSlides were incubated with microtubuli stabilizing buffer (1x MTSB) for 5 min to maintain the architecture of the cytoskeleton. After fixing the cells with 4% paraformaldehyde (PFA) for 20 min they were permeabilized with 1% Triton X-100 (TX-100) for 5 min and blocked with 5% BSA for at least 1 h. Primary rabbit anti-OctA-Probe-antibody (1:500 in 1% BSA, Santa Cruz Biotechnology) and/or mouse anti HA-antibody (1:250 in 1% BSA, Santa Cruz Biotechnology) were incubated overnight at 4 °C. After three washing steps (1x PBS) secondary Alexa Fluor 488-labelled goat anti-rabbit IgG (1:250 in 1% BSA, Life Technologies) and/or 555-labelled goat anti-mouse IgG (1:250 in 1% BSA, Life Technologies) was incubated for 1 h before mounting with ProLong Gold Antifade Reagent (Life Technologies). Confocal microscopy was performed with a LSM 510 Meta and a 63x/1.4 Oil lens (Zeiss) and pictures were modified using LSM Image Browser (Zeiss). Displayed pictures are representatives of five independent experiments.

Total-protein Extraction
For total-protein extracts HEK-293T cells were detached with ice-cold 1x PBS and centrifuged for 5 min at 200 g (4 °C). Pelleted cells were washed once with 1x PBS. Pellets were resuspended in lysis buffer (120 mmol/L NaCl, 25 mmol/L Hepes (pH 7.5), 1% TX-100, 2 mmol/L EDTA, 25 mmol/L NaF, 1 mmol/L sodium orthovanadate, 0.2% SDS, 1 mmol/L PMSF and 1x Complete Mini EDTA-free Cocktail tablet) and incubated for 15 min on ice. Lysates were centrifuged at 13,000 g at 4 °C for 15 min.
Western Blot
Lysates were heated at 95 °C for 5 min in a 2x sample buffer (Laemmli buffer, Biorad). Equal amounts of the samples were subjected to electrophoresis and separated on Any kD™ Mini-Protean® TGX™ Precast Gels (Biorad) according to manufacturer’s protocols. Gels were blotted to 0.2 µm nitrocellulose membranes using the Trans-Blot Turbo Blotting System (Biorad) for mixed molecular weights (1.3 A, 25 V, 7 min). The membrane was equilibrated in 1x TBS and blocked with 5% non-fat milk powder. Primary antibodies (Anti-HA-Probe (F7) mouse monoclonal Antibody (1:100) and Anti-OctA-Probe (D-8) rabbit polyclonal Antibody (1:200); respectively Anti-Gβ2 (C-16) rabbit polyclonal Antibody (1:100) and Anti-GFP (B-2) mouse monoclonal Antibody (1:1000), all obtained from Santa Cruz Biotechnology, were incubated overnight in 1x TBS. After three washing steps with 1x TBS-Tween 20, secondary horseradish peroxidase (HRP)-labelled secondary antibodies (ECL Anti-mouse IgG, ECL Anti-rabbit IgG, 1:5000 in 1x TBS, GE Healthcare) were incubated for 1 h followed by washing three times with 1x TBS-Tween 20. Protein staining was visualized using Western Blotting Luminol Reagent (Santa Cruz) and membrane exposure to FUSION-SL chemiluminescence system (Peqlab). Densitometric analysis of Western Blots was performed by BIO-1D-Software (Peqlab).

Analysis of Gβ2-Arg52Leu stability
To check the stability of the Gβ2-Arg52Leu protein, HEK-293T cells were transfected with pIRES-acGFP-GNB2-Flag or pIRES-ac-GNB2-Arg52Leu-Flag and pcDNA3.1-GNG2 (2 µg each) using 6 µl x-tremeGENE 9 Transfection Reagent (Roche) in 100 µl Opti-MEM per dish. At 48 h after transfection, the cells were treated with 100 µg/ml cycloheximide for 0, 2, 4, 8 and 24 hours. Total-protein cell extracts were analyzed by Western blot. Quantification of protein amounts were obtained by dividing volumes of individual Gβ2 bands through corresponding GFP bands.

Two-electrode voltage clamp (TEVC) recordings of Kir3.1/Kir3.4 mediated currents in presence of G-proteins
For cRNA synthesis and electrophysiological measurements in *Xenopus laevis* oocytes wild type (NM_005273.3) and mutant GNB2, native GNG2 (NM_001243774), KCNJ5 (Kir3.4; NM_000890.3) and KCNJ3 (Kir3.1; NM_002239) cDNAs were subcloned into the oocyte expression vector psGEM. After vector linearization with NheI cRNA synthesis for subsequent injection into oocytes was performed with T7 mMessage mMachine Kit (Ambion Inc) following manufacturer’s protocol. The quality and quantity of cRNA was verified using NanoDrop® (Peqlab).

The *Xenopus laevis* oocytes (EcoCyte Bioscience) were injected with human Kir3.1/3.4 cRNA (2 ng each), human Gβ2 or Gβ2-Arg52Leu cRNA (5.1 ng) and Gγ2 cRNA (5.1 ng). The oocytes were incubated for 72 h at 18 °C in Barth’s solution containing 88 mmol/L NaCl, 1.1 mmol/L KCl, 2.4 mmol/L NaHCO3, 0.3 mmol/L Ca(NO3)2, 0.4 mmol/L CaCl2, 0.8 mmol/L MgSO4, 15 mmol/L HEPES, 63 µg/ml penicillin-G, 40 µg/ml streptomycin sulfat, 80 µg/ml theophylline and 100 µg/ml gentamycin (pH 7.6).

Two-electrode voltage-clamp recordings were performed at 22 °C using the TurboTec-10CD (NPI) equipped with an AD/DA-interface ITC-16 (HEKA Elektronik). Data analyses were done with Pulse/Pulsefit (HEKA Elektronik) and OriginPro 8.5.1 SR (OriginLab Corporation). Recording pipettes were filled with 3 M KCl and had a resistance of 0.4 - 1.5 MΩ. Ion currents were recorded 72 h after injection in a KD60 recording solution (32 mmol/L NaCl, 60 mmol/L KCl, 1.8 mmol/L MgCl2, 1.0 mmol/L CaCl2 and 5 mmol/L HEPES, pH 7.6) and in a ND96 recording solution (96 mmol/L NaCl, 4 mmol/L KCl, 1.8 mmol/L MgCl2, 1.0 mmol/L CaCl2 and 5 mmol/L HEPES, pH 7.4). Oocytes were held at 0 mV and ion currents were elicited with 2000 ms pulses to potentials from -140 mV to +60 mV in +20 mV increments.

Two-electrode voltage clamp (TEVC) recordings of **Cav1.2, HCN2 or HCN4 channels in presence of Gβ2**
*Xenopus* oocytes were isolated as previously described¹ and stored at 18 °C in ND96 recording solution (containing in mmol/L): NaCl 96, KCl 2, CaCl2 1.8, MgCl2 1, HEPES 5; pH 7.4 with NaOH, supplemented with Na-pyruvate (275 mg/l), theophylline (90 mg/l) and gentamycin (50 mg/l). The
Cav1.2, Cavβ2b and Cavα2δ long variant clones used in this study were previously described. Cav1.2 channels were subcloned in pBS, linearized with NotI and cRNA was synthesized using T7 polymerase. Cavβ2b was subcloned in pSPORT, linearized with NotI and cRNA was made with T7 polymerase. Cavα2δ long variant clone was subcloned in pSP65, linearized with SalI and cRNA was made with SP6 polymerase. Linearization of GNG2, wild type or mutant GNB2 subcloned in pSGEM, was performed as described above and cRNA was synthesized using T7 polymerase (HiScribe T7 ARCA mRNA Kit, New England BioLabs). For TEVC recordings of Cav1.2, stage IV and V oocytes were injected with 11 ng of human Cav1.2 and 1.35 ng rabbit Cavβ2b and Cavα2δ long cRNAs each. For co-expression experiments additionally 5 ng Gγ2 and 5 ng human Gβ2 or Gβ2-Arg52Leu cRNA were used. After 48 h standard two-electrode voltage clamp (TEVC) experiments were performed at room temperature (21 - 22 °C) in calcium channel recording solutions (containing in mmol/L): BaOH2 40, NaOH 50, KOH 1, HEPES 5, pH 7.4 with methanesulfonic acid. Niflumic acid (300 µM) was added to the solution to block Ca2+-activated Cl- currents. Agar bridges were used for these recordings. Each oocyte was washed in four consecutive baths of recording solution prior to measurement to rinse from ND96. For HCN2 and HCN4 TEVC recordings stage IV and V oocytes were co-injected with 5.1 ng of HCN2 or 25.3 ng of HCN4 cRNA and 5.1 ng of Gγ2 together with either 5.1 ng of human Gβ2 or Gβ2-Arg52Leu cRNA. Voltage clamp recordings were performed in ND66 solution (containing in mmol/L): NaCl 66, KCl 32, CaCl2 1.8, MgCl2 1, HEPES 5; pH 7.5 with NaOH, 2-3 days after injection of the respective constructs. IV-curves were recorded by voltage steps of 6 s duration to potentials ranging from -30 mV to -140 mV from a holding potential of -30 mV. Tail currents were recorded by a 1 s test pulse to -130 mV. Microelectrodes were fabricated from glass capillary tubes and filled with 3 M KCl. Tip resistance was in the range of 0.5 - 1.0 MΩ. TEVC recordings were performed using a TurboTEC-10CD Amplifier (npi) with a Digidata 1200 A/D-converter (Axon Instruments). For data acquisition the software pCLAMP7 (Axon Instruments) was used. Data were analyzed with ClampFit10 (Axon Instruments).

**Animals**
The investigation conforms to the guide for the Care and Use of laboratory Animals (NIH Publication 85-23). For this study four female *Xenopus laevis* animals were used to isolate oocytes. Experiments using *Xenopus* toads were approved by the local ethics commission of the “Regierungspräsidium Gießen”.

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### Online Tables

**Online Table I: Primer sets used for qPCR.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence 5' - 3'</th>
</tr>
</thead>
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| GAPDH | Forward: CAGCCTCAAGATCATCAGCA  
Reverse: GTCTTCTGGTGTCAGTGAT |
| GJA5  | Forward: AGAGTGTGAAGAGGCCCACG  
Reverse: TTCCAGAGACCTCAATCG |
| TBX3  | Forward: GTGCACCTGGAGGCTAAGA  
Reverse: TCCAGCCAGAACATCTCAC |
| NPPA  | Forward: TGACGTTCCTCTTTTACTGG  
Reverse: TTCTTCAATGGATCCAGC |
| HCN4  | Forward: GATCCTCAGCCTTTACGCC  
Reverse: ATCATGCCAGTGAGGTCAG |
| GNB1  | Forward: GCAACTCTCTCTCAGATCACAAAAC  
Reverse: CACTGACGAGAAGCCTGGAG |
| GNB2  | Forward: AGATCACAGCTGGCTG  
Reverse: GATGAGCTTCCCATCCTACG |
| GNB3  | Forward: CTGTGCTGACGTACTTCTG  
Reverse: ATCTGGACTTCCCATCTAG |
| GNB4  | Forward: GAGGCAAGAAGCAGAACAACGTG  
Reverse: CATTTGTATTGCACCCACGGAG |
| GNB5  | Forward: GTGTGATGCTGATTATATGC  
Reverse: TTGGTGATGCTAGCACAACAG |
| GNG2  | Forward: AGGATAAAGGTGTCGAAGGC  
Reverse: CAAAGACTTAAAGGATGGCACAG |
| GNG7  | Forward: AAAGCTCTCTGAACGAGG  
Reverse: AGACCTTGATGCTCGAATTC |
| GNG12 | Forward: GCAAGGAGAAGCTGTCAGCAG  
Reverse: GGATTTCTATCGCAAGGAGTTC |
**Online Table II:** LOD scores in the chromosome 7q21.1-q31.1 candidate region calculated in parametric two-point analysis.

<table>
<thead>
<tr>
<th>Microsatellite Locus</th>
<th>LOD Score at Recombination Fraction (θ)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td>D7S630</td>
<td>-∞</td>
</tr>
<tr>
<td>D7S2410</td>
<td>-∞</td>
</tr>
<tr>
<td>D7S646</td>
<td>-1.05</td>
</tr>
<tr>
<td>D7S657</td>
<td>0.60</td>
</tr>
<tr>
<td>D7S1820</td>
<td>1.29</td>
</tr>
<tr>
<td>D7S2482</td>
<td>1.93</td>
</tr>
<tr>
<td>D7S821</td>
<td>-∞</td>
</tr>
<tr>
<td>D7S491</td>
<td>4.54</td>
</tr>
<tr>
<td>D7S2480</td>
<td>4.64</td>
</tr>
<tr>
<td>D7S515</td>
<td>2.83</td>
</tr>
<tr>
<td>D7S501</td>
<td>4.34</td>
</tr>
<tr>
<td>D7S471</td>
<td>4.64</td>
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<tr>
<td>D7S2502</td>
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<tr>
<td>D7S677</td>
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</tr>
<tr>
<td>D7S643</td>
<td>-∞</td>
</tr>
<tr>
<td>D7S650</td>
<td>-∞</td>
</tr>
<tr>
<td>D7S1873</td>
<td>-∞</td>
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<tr>
<td>D7S2501</td>
<td>-∞</td>
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<tr>
<td>D7S530</td>
<td>-∞</td>
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</table>
Online Table III: List of validated gene variants on chromosome 7q21.1-q31.1 identified after targeted exome sequencing of the family’s proband (8).

<table>
<thead>
<tr>
<th>Gene</th>
<th>OCM2</th>
<th>GNB2</th>
<th>SRPK2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NM_005273.3</td>
<td>NM_182692.2</td>
</tr>
<tr>
<td>Protein</td>
<td></td>
<td>G-protein subunit β2</td>
<td>serine/arginine-rich protein specific kinase 2</td>
</tr>
<tr>
<td>Variant [c.]</td>
<td>59A&gt;G</td>
<td>155G&gt;T</td>
<td>35G&gt;A</td>
</tr>
<tr>
<td>Variant [p.]</td>
<td>(Gln20Arg)</td>
<td>(Arg52Leu)</td>
<td>(Arg12Gln)</td>
</tr>
<tr>
<td>MAF in EVS [%]</td>
<td>absent</td>
<td>absent</td>
<td>absent</td>
</tr>
<tr>
<td>MAF in ExAC [%]</td>
<td>absent</td>
<td>absent</td>
<td>absent</td>
</tr>
<tr>
<td>PolyPhen-2</td>
<td>benign</td>
<td>probably damaging</td>
<td>possibly damaging</td>
</tr>
<tr>
<td>SIFT</td>
<td>tolerated</td>
<td>deleterious</td>
<td>tolerated</td>
</tr>
<tr>
<td>Mutation taster</td>
<td>disease causing</td>
<td>disease causing</td>
<td>polymorphism</td>
</tr>
<tr>
<td>MutPred</td>
<td>neutral</td>
<td>deleterious</td>
<td>neutral</td>
</tr>
<tr>
<td>CADD Score</td>
<td>14.31</td>
<td>24.8</td>
<td>26.4</td>
</tr>
<tr>
<td>Familial co-segregation</td>
<td>no</td>
<td>yes</td>
<td>no</td>
</tr>
</tbody>
</table>
Online Table IV: Contact analysis in YASARA structure after molecular dynamic simulations revealed contacts (4 Å cut off) of GNB2 residues Arg52 (Gβ2 native) or Leu52 (Gβ2 mutant) with the GIRK channel subunits (Kir3.1 + Kir3.4). Listed are contacts of residue 52 in the individual Gβ2 subunit with GIRK.

<table>
<thead>
<tr>
<th>Gβ2 subunit</th>
<th>Kir 3.1 subunit</th>
<th>Kir 3.4 subunit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Contacts of residue</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gβ2 native</td>
<td>Gβ2 mutant</td>
</tr>
<tr>
<td>1</td>
<td>K48 (n=2)</td>
<td>E45 (n=5)</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>E340 (n=5)</td>
</tr>
<tr>
<td></td>
<td>L339 (n=6)</td>
<td>K341 (n=7)</td>
</tr>
<tr>
<td>2</td>
<td>K48 (n=2)</td>
<td>E45 (n=5)</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>E340 (n=6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L339 (n=6)</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>G342 (n=1)</td>
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<tr>
<td></td>
<td>E340 (n=6)</td>
<td>E340 (n=6)</td>
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<tr>
<td></td>
<td>L339 (n=7)</td>
<td>L339 (n=9)</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>K341 (n=1)</td>
</tr>
<tr>
<td></td>
<td>E340 (n=4)</td>
<td>E340 (n=1)</td>
</tr>
<tr>
<td></td>
<td>L339 (n=8)</td>
<td></td>
</tr>
</tbody>
</table>
Online Figures

A. Representative precordial ECG leads (paper speed 50 mm/s) of patient 8 at age 38 years (paper speed 50 mm/s). Bradycardia, irregular P-P (marked by arrow) and R-R intervals, prolonged AV conduction (>200 ms; dashed line), but without typical Wenckebach pattern. Binodal disease.

B. Representative limb ECG leads (paper speed 50 mm/s) of patient 8 at age 52 years (paper speed 50 mm/s). Continuous ventricular pacing, intermittent atrial pacing; post-pacing PVB (*).

C. Baseline ECG (paper speed 50 mm/s) of patient 13 at age 47 years. Atrial fibrillation (68/min), also bradycardic.

Online Figure IA-C. ECG characteristics of GNB2 R52L mutation carriers.
A. Representative precordial ECG leads (paper speed 50 mm/s) of patient 8 at age 38 years (paper speed 50 mm/s). Bradycardia, irregular P-P (marked by arrow) and R-R intervals, prolonged AV conduction (>200 ms; dashed line), but without typical Wenckebach pattern. Binodal disease.
B. Representative limb ECG leads (paper speed 50 mm/s) of patient 8 at age 52 years (paper speed 50 mm/s). Continuous ventricular pacing, intermittent atrial pacing; post-pacing PVB (*).
C. Baseline ECG (paper speed 50 mm/s) of patient 13 at age 47 years. Atrial fibrillation (68/min), also bradycardic.
Online Figure ID, E. ECG characteristics of GNB2 R52L mutation carriers.
D. Exercise ECG (paper speed 10 mm/s) of patient 20 at age 18 (paper speed 50 mm/s). Initially, continuous ventricular or AV pacing; at 100W (left), intrinsic AV conduction without pacing. At high endurance levels (225W, right), mostly single PVB.
E. Baseline ECG (paper speed 50 mm/s) of patient 22 at age 18. Sinus bradycardia, 42/min, regular P-P and R-R intervals, normal AV conduction.
Online Figure II:
A. Relative abundance for mRNA of cardiac marker genes. *NPPA* is a positive marker for atrial tissue, whereas *TBX3*, *GJA5* and *HCN4* are preferentially expressed in tissue of the conduction system.
B. Relative abundance of mRNA for *GNB1-5* and *GNG2, GNG7, GNG12* in human RNA samples obtained from different heart tissues and brain.
A=atrium, V=ventricle, SN=sinus node, AV=atrioventricular node, PF=Purkinje fibres, B=brain.
Data are provided as mean ± SEM
Online Figure III:
A. Time course of native and mutant Gβ2 degradation.
Left Panel: Representative Western Blot analysis of cell lysate from HEK-293T cells expressing either native Gβ2 or mutant Gβ2-Arg52Leu at different time points after treatment with 100 µg/ml cyclohexamide. Cell lysates (10 µl) were analyzed and probed with the anti-GNB2 and anti-GFP antibody as a transfection and internal loading control.
Right panel: Degradation rates of native and mutant Gβ2 normalized to GFP. The graphs represented the averages from four independent experiments and error bars represent the standard error of the mean (SEM).

B. Confocal immunofluorescence of the Gβ2 and Gγ2 protein expression in COS-7 cells.
COS-7 cells were co-transfected with wild type or flagged tagged Gβ2-Arg52Leu and with pDsRed-Monomer-F Hyg- (Plasma membrane) or HA-tagged Gγ2. Double immunostaining of Gβ2 and the plasma membrane (PM) (left panel) or Gγ2 (right panel). The subcellular localisation of the Gβ2-Arg52Leu protein is not significantly different from native Gβ2 protein, since both, Gβ2 as well as Gβ2-Arg52Leu localize to the plasma membrane and co-localize with the Gγ2 protein.
Online Figure IV:
Gβ2 and Gβ2.Arg52Leu induced activation of Kir3.1/3.4 in *Xenopus laevis* oocytes. The oocytes were injected with Kir3.1 and Kir3.4 (each 2 ng) alone or with native Gβ2/mutant Gβ2.Arg52Leu + Gγ2 (each 5.1 ng). Currents (measured at the end of the pulse) were normalized to the GIRK current at -140mV.
A. Representative Kir3.1/3.4 current traces elicited by a pulse protocol in KD60.
B. Current-voltage relationship measured using a pulse protocol in KD60.
C. Representative Kir3.1/3.4 current traces elicited by a pulse protocol in ND96.
D. Current-voltage relationship measured using a pulse protocol in ND96. Data are provided as mean ± SEM. P values calculated in unpaired Student’s t-test refers to the Kir3.1/3.4 Gβ2.
Online Figure V:

G-protein modulation of native Cav1.2, HCN2 and HCN4 channels in presence of native/mutant Gβ2 + native Gγ2.
A. Representative current traces recorded in *Xenopus* oocytes after co-expression of Cav1.2 with Cavβ2b and Cavα2δ subunits (black) alone or after additional co-expression with Gβ2 and and Gγ2 (blue) or Gβ2-Arg52Leu and Gγ2 (red). Currents were recorded from a holding potential of -80 mV, with voltage steps of 1 s duration ranging from -80 to +60 mV in 10 mV increments. Sweep time interval was 30 s.
B. Current voltage relationships after cRNA injection as in (A).
C. Analysis of the relative peak current amplitudes, normalized to Cav1.2 expressed with Cavβ2b and Cavα2δ subunits (black).
D. Normalized current-voltage relationship of recordings with less than 5 µA of amplitude.
E. Representative current-voltage relationship recordings of HCN2 co-expressed with Gγ2 together with Gβ2 or Gβ2-Arg52Leu, and
F. relative current amplitudes analyzed at -120 mV.
G. Representative current-voltage relationship recordings of HCN4 expressed with Gγ2 together with Gβ2 or Gβ2-Arg52Leu.
H. The relative current amplitudes analyzed at -120 mV.
The numbers of experiments are indicated within the bar graph. Data are presented as mean ± SEM. Significance was probed using ANOVA. **, p ≤ 0.01.
References

