Life Sciences 219 (2019) 74–81

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Preprodynorphin gene mutation causes progressive cardiac conduction disease: A whole-exome analysis of a pedigree

Jian-Yao Su[a], Rong-Feng Zhang[a], Ying-Xue Dong[a], Ming-Hui Yang[a], Xiao-Meng Yin[a], Lian-Jun Gao[a], Hui-Hua Li[b], Yun-Long Xia[a,*], Yan-Zong Yang[a,*]

[a] Department of Cardiology, The First Affiliated Hospital of Dalian Medical University, Dalian 116011, China
[b] Department of Cardiology, Institute of Cardiovascular Disease, The First Affiliated Hospital of Dalian Medical University, Dalian 116011, China

ARTICLE INFO

Keywords:
Progressive cardiac conduction disease
PDYN
Whole-exome sequencing
Gene mutation
Familial

ABSTRACT

Aims: Progressive cardiac conduction disease (PCCD) is a rare heart disease that usually shows familial inheritance. Potential genetic risk factors for PCCD have been mostly limited to genes that encode ion channels, cardiac transcription factors, T-box transcription factors, gap junction proteins, energy metabolism regulators and structural proteins.

Main methods: Subjects in the present study came from a family who exhibited the autosomal dominant inheritance of PCCD. The primary proband had syncope and an electrocardiogram typical for PCCD, which started in the left bundle branch block, and passed to the atrioventricular block. The patient received a permanent pacemaker in 2013. Pathogenic mutations in the proband's family were identified using whole-exome sequencing and Sanger sequencing.

Key findings: The results for the family members were verified using Sanger sequencing, while the results for healthy unrelated individuals were verified using SNaPShot. All patients in the family shared two adjacent missense mutations in the preprodynorphin (*PDYN*) gene (c.581A > T, c.580G > C; p.D194L).

Significance: The *PDYN* double mutation c.581A > T and c.580G > C (p.D194L) may be linked to the onset of familial PCCD. The effects of these mutations on electrophysiology require further investigation.

1. Introduction

Progressive cardiac conduction disease (PCCD, MIM #113900) is a rare heart condition with a genetic basis that usually shows familial inheritance. PCCD has been less studied, when compared to other hereditary heart diseases, but several genes have already been implicated in progressive conduction system disorders [1,2]. These genes encode ion channels, such as voltage-gated sodium channel α-subunit (SCN5A) and β-subunit (SCN1B), voltage-gated sodium channel alpha Nav1.8 (SCN10A), hyperpolarized activated cyclic nucleotide-gated potassium channel 4 (HCN4), inward-rectifier potassium ion channel (KCNJ2), transient receptor potential cation channel subfamily M member 4 (TRPM4), and tandem pore domain potassium channel (KCNK17). Other genes have been implicated, including genes encoding cardiac transcription factor Homeobox protein Nkx-2.5 (NKX2.5), T-box transcription factor TBX5, gap junction protein connexin 40 (Cx40), energy metabolism regulator PRKAG2, and structural protein lamin A/C (LMNA). In the present study, whole-exome sequencing was applied, and mutations in the prodynorphin (*PDYN*) gene were identified in a family with PCCD. These mutations constitute a novel etiologic factor of PCCD.

PDYN is a precursor of dynorphins (Dyn), which is an endogenous opioid that binds to the κ-opioid receptor (KOR), and is the predominant opioid receptor subtype in the cardiovascular system. Other endogenous opioids, endorphins and enkephalins, bind to δ- and μ-opioid receptors, respectively. Dyn and other opioid peptides reach the heart by being released from the central nervous system, and enter the systemic circulation by being released from peripheral nerve endings, or by being synthesized within the heart itself [3–5]. The myocardium upregulates the synthesis and release of opioid peptides in response to ischemia [6,7], exercise [8] and cardiac intervention [9], and the extent of this upregulation is affected by age [3] and heart failure [10,11]. The expression of opioid peptides and the corresponding receptors can affect myocardial response, and thereby leads to tolerance to ischemia and reperfusion [6,7,12].

The activation of the opioid system appears to increase the

* Corresponding authors at: Department of Cardiology, The First Affiliated Hospital of Dalian Medical University, No. 222 of Zhongshan Road, Xigang District, Dalian 116011, China.
E-mail addresses: yunlong_xia@126.com (Y.-L. Xia), yanzongyangdr@163.com (Y.-Z. Yang).

https://doi.org/10.1016/j.lfs.2018.12.062
Received 14 September 2018; Received in revised form 18 December 2018; Accepted 31 December 2018
Available online 03 January 2019
0024-3205/

resistance to oxidative stress and ischemia, at least in part, by opening mitochondrial ATP-sensitive K^+ channels (K^+_{ATP}), thereby inducing mitochondrial swelling [13], which may be beneficial to the cardiac muscle [14]. It is possible that the opioid system also affects the cardiac conduction system. For example, κ-selective agonists stimulate arrhythmogenic activity in isolated perfused rat hearts [15]. The present results link PDYN to PCCD, providing evidence that the perturbation of the opioid system can lead to cardiac conduction disease.

2. Materials and methods

2.1. Ethics statement

The study protocol was approved by the Ethics Committee of the First Affiliated Hospital of Dalian Medical University, China. This study was conducted in accordance with the Declaration of Helsinki, and a written informed consent was obtained from all PCCD patients and healthy participants.

2.2. Pedigree analysis and clinical evaluation

2.2.1. Study population

The present study involved 34 individuals spanning four generations of a family with PCCD (Fig. 1A). Genomic DNA was isolated from venous blood collected from each living member of the PCCD pedigree. The results from this pedigree analysis were compared with results obtained from 410 unrelated healthy individuals, who were recruited at the First Affiliated Hospital of Dalian Medical University.

2.2.2. Clinical assessment

The pedigree analysis was conducted to analyze the hereditary pattern of PCCD transmission. The disease conditions of the immediate and extended family members of the patients were determined through the review of hospitalization records and follow-up survey of the pedigree members. The results were represented as a pedigree chart (Fig. 1A). The basic information about the family members, including demographic data and medical history, were recorded, and electrocardiography and ultrasonic cardiography were performed.

Individuals were diagnosed with PCCD primarily on the basis of medical history, family history, the results of the 12-lead electrocardiography, and other clinical data. Usually, patients were diagnosed with PCCD when they have a family history of cardiac conduction disease, were younger than 50 years, and presented with a normal heart structure and no skeletal muscle disease, regardless of the presence of inducible factors for progressive disorders of the heart conduction system [16–18]. The ultrasonic cardiography can exclude congenital heart disease and potential myocardial disease.

2.3. Whole-exome sequencing

Genomic DNA was extracted from peripheral blood leukocytes using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). The DNA in exons of the whole genome was captured, enriched and analyzed using high-throughput sequencing. All genomic DNA samples were selected from three patients in the pedigree for whole-exome sequencing. A DNA library was established using a TruSeq Exome Enrichment Kit (Illumina, San Diego, CA, USA), and sequenced on a HiSeq2000 (Illumina) platform. All exons of all genes were amplified to at least at 30×. High-quality reads were obtained from the raw data, and were compared with the human reference genomic sequence (hg19). Then, single-nucleotide variations, and the insertions/deletions of small fragments were detected. All these changes were annotated and classified using Annovar software. The variant filtering process was designed to identify very rare amino acid changing variants (i.e. missense, nonsense, structural variants, or splice site variants). All variations detected in the whole-exome sequencing analysis were queried and compared against databases (dbSNP), the 1000 Genomes Project, and the Exome Sequencing Project (ESP). PCCD is an autosomal dominant inheritance. Therefore, other types of inheritances (i.e. Autosomal recessive inheritance and Sex chromosome inheritance) were excluded. The Sanger sequencing primers are listed in Table 1.

2.4. Annotation analysis

2.4.1. Sanger sequencing for screening within the pedigree

The aim of the present study was to identify pathogenic mutations in a family with PCCD. Since this disease shows an autosomal dominant pattern of inheritance, it was expected that disease-related mutations in the pedigree should appear only in patients, and not in unaffected family members. Sanger sequencing was performed to analyze candidate pathogenic mutations in other living patients in the family (III.8), as well as in other family members. Any variations shared by both patients should be linked to PCCD.

Genomic DNA was amplified by polymerase chain reaction (PCR), and the amplification products were sequenced using a BigDye Mix kit onan ABI 3730 sequencer (Applied Biosystems, Foster City, CA, USA). The PCR reaction (5 μL) contained 1 μL (3.2 μM) of each unidirectional primer, 2 μL of purified PCR product, and 1 μL of BigDye Mix. The PCR reaction conditions were as follows: denaturation at 94 °C for 1 min; 28 cycles of 94 °C for 20 s, 50 °C for 10 s, and 60 °C for 4 min; finally, incubation at 4 °C. The PCR products were purified and pretreated prior to sequencing.

2.4.2. SNaPshot sequencing for mutation validation

SNaPshot sequencing was performed on genomic DNA from 410 unrelated control individuals. The upstream primer used to amplify the *PDYN* variant c.581A > T was TCTGAGCTGATGAGGGATGC, the corresponding downstream primer was GTTGTCCCACTTGAGCTTGG, and the extension primer was TTTTTTTTTTCTCATGGCCCATGCTATCC CCG. The upstream primer to amplify the *PDYN* variant c.580G > C was TCTGAGCTGATGAGGGATGC, the downstream primer was GTTG TCCCACTTGAGCTTGG, and the extension primer was TTTTTTTTTTT TTCTCAGAGGTGGCTGGGGAGGGG.

The PCR reaction contained 1 μL of DNA, 1.5 μL of 10× buffer, 1.5 μL of $MgCl_2$ (25 mmol), 0.3 μL of dNTPs (10 mmol), 0.15 μL of primers (10 μmol), 0.3 μL of Taq enzyme (5 U/μL), and H_2O in a total volume of 15 μL. The reaction conditions were as follows: 94 °C for 3 min; 35 cycles of 94 °C for 15 s, 55 °C for 15 s, and 72 °C for 30 s; finally, 72 °C for 3 min. After PCR amplification, 3 μL of the PCR products were purified using 0.2 μL of Exo I (20 units/μL), 0.8 μL of FastAP (1 U/μL), 0.7 μL of Exo I buffer, and 7 μL of H_2O. The reaction conditions were as follows: 37 °C for 15 min and 80 °C for 15 min. The extension reaction was immediately performed after purification. A total of 1 μL of the extension product was added to 9 μL of loading buffer (HIDI formamide, Applied Biosystems, Foster City, CA), and the samples were denatured at 95 °C for 3 min. Immediately thereafter, the samples were placed in an ice bath and loaded into a sequencer. Ten samples were randomly selected for Sanger sequencing to verify the accuracy of the SNaPshot sequencing.

3. Results

3.1. Clinical data and pedigree

Proband III.3 was a 45-year-old male who underwent a 24-h Holter examination in 2003 at the First Affiliated Hospital of Dalian Medical University due to palpitations. The examination revealed frequent premature atrial contraction, paroxysmal atrial tachycardia, and occasional premature ventricular contraction. In 2007, the proband had recurrence of heart palpitations with more severe symptoms and more frequent episodes, and was admitted to the hospital, where he underwent electrocardiography and 24-h Holter examination several times.

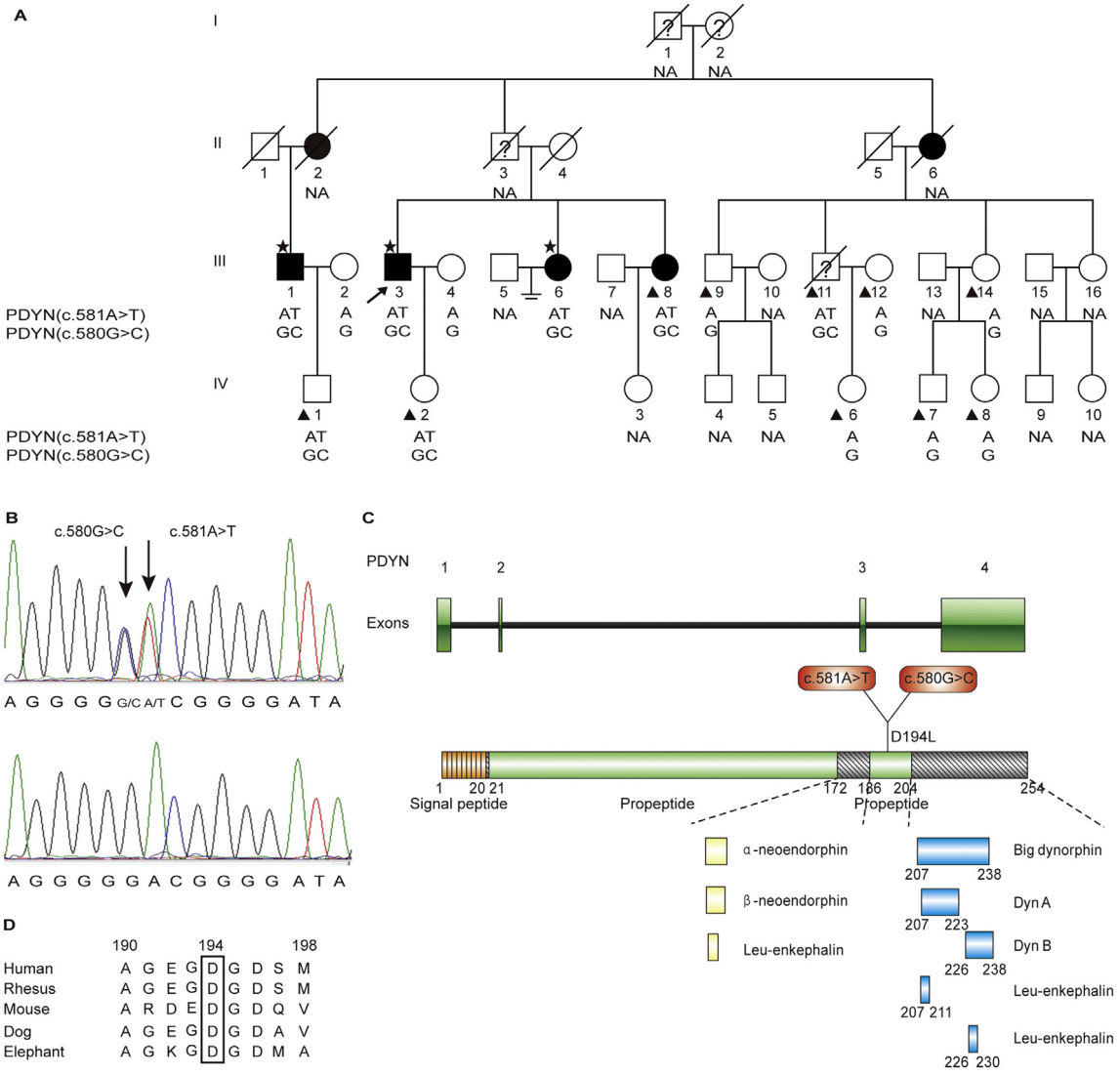

Fig. 1. Mapping analysis of a family with PCCD, which identified the missense *PDYN* mutations c.581A > T and c.580G > C (p.D194L). (A) Individuals with PCCD caused by mutations in the *PDYN* gene. Squares indicate male family members; circles, female family members; slashes, deceased family members; arrows, the proband; solid symbols, patients with cardiac conduction disease; open symbols, unaffected family members. Question marks indicate that the individual's condition was not determined. Two mutations in *PDYN* (c.581A > T and c.580G > C) were identified in seven individuals (III.1, III.3, III.6, III.8, III.11, IV.1, and IV.2). Triangles indicate Sanger sequencing. Pentagons indicate Whole-exome sequencing. AT and GC indicate the mutations, while A and T indicate wild-type genotypes. "NA" designates members of the pedigree who refused to provide blood samples. (B) Sanger sequencing electropherograms showing two *PDYN* mutations (c.581A > T and c.580G > C) within a single codon on the same allele. (C) *PDYN* exons 3 and 4 encode PDYN, which yields the opioid peptides α-neoendorphin (α-NE), β-neoendorphin (β-NE), leu-enkephalin, dynorphin A (Dyn A), dynorphin B (Dyn B), and big dynorphin (Big Dyn), which encompasses Dyn A and Dyn B. The missense *PDYN* mutations linked to PCCD in the present study (c.581A > T, c.580G > C) result in a substitution of Asp194 to Leu (D194L). (D) The Asp194 that was mutated in PCCD patients in our pedigree is conserved among species.

Table 1
Primers applied in Sanger sequencing.

Gene	cDNA change	Forward primer	Reverse primer
ADAMTS4	c.920C > T	TGGTGGCAGATGACAAGATGG	ACCTGACGGGTAAACAGAATG
CHIT1	c.543T > G	AACATCTACCTGGGGCTCACA	GTTCTGGTGTAAGGATGGGAC
MFF	c.893G > A	TGAGCATTACACAAAGCATGATG	TTTCAAAAGCCAAACAAGCAA
PTPN13	c.416T > A	TATTTGCTCACATTTACTCAC	ATTTCCCAGAACCAGTTTTAC
MANBA	c.530A > G	CGTTTCCAGTCAGCGGTGTTG	TAAAGTTCCATTATCAGCCAC
CYB5R2	c.416C > T	TGGTGCAGACAGTCCCTTAG	CTGGATGGAGCCCCTAGAAG
ANKRD11	c.5221G > A	CCTACGTCGGTGCTATCCTG	AAGGTTTGTGGAGAGAGGCC
PDYN	c.581A > T	TCTGAGCTGATGAGGGATGC	GTTGTCCCACTTGAGCTTGG
PDYN	c.580G > C	TCTGAGCTGATGAGGGATGC	GTTGTCCCACTTGAGCTTGG

Table 2
Clinical characteristics of patients with PCCD in this study.

Clinical characteristics								Echocardiography		
ID	Sex	Age	Age at conduction diagnosis	Conduction disease	Syncope	Atrial arrhythmia	PPM	LVEF (%)	LA (mm)	LV (mm)
II. 2	F	NA[a]	39	III°AVB CLBBB	+	AF	+	NA	40	45
II. 6	F	NA[a]	NA	SB CRBBB	NA	PAC, AT	−	34	47	62
III. 1	M	55	45	III°AVB CRBBB	+	AF	+	58	43	60
III. 3	M	45	43	LBBB,SB I/II/III°AVB	+	PAC, AT	+	58	42	53
III. 6	F	52	48	III°AVB	+	AF	+	60	45	50
III. 8	F	51	49	I°AVB	−	−	−	60	37	49

Note: AF, atrial fibrillation; AT, atrial tachycardia; AVB, atrioventricular block; CRBBB, complete right bundle branch block; F, female; ID, subject index; LA, left atrium; LBBB, left bundle branch block; LV, left ventricle; LVEF, left ventricular ejection fraction; M, male; NA, not available or not applicable; PAC, premature atrial contraction; PPM, permanent pacemaker; SB, sinus bradycardia; +, present; −, absent.
[a] Age at death.

These tests revealed paroxysmal atrial tachycardia and premature ventricular contraction, with a wide left bundle branch block. In 2013, the proband was again admitted to the hospital due to chest tightness and worsened palpitations after heavy drinking. The electrocardiography and 24-h Holter examination revealed that the total number of heartbeats in 27 h and 48 min was 78,317, the slowest heart rate was 32 bpm, and the fastest heart rate was 98 bpm. These tests also revealed sinus bradycardia and a paroxysmal degree I-III atrioventricular block. The proband exhibited syncope during this period, and underwent permanent pacemaker implantation in 2013.

Neither of the proband's parents was alive at the time that the proband was treated, and neither had a history suggestive of PCCD. Further investigation led to a diagnosis of PCCD in the proband's sibling III.1 and III.8, as well as in two of the five cousins (Fig. 1A). The proband's two aunts were suspected of PCCD, based on the review of medical records at the First Affiliated Hospital of Dalian Medical University. The clinical features included sinus bradycardia, atrioventricular block of varying degrees, premature atrial contraction, atrial tachycardia, and atrial fibrillation (Table 2). Four members of the family received permanent pacemaker implantation. All 10 persons in the next generation appeared to be healthy. A preliminary analysis of the pedigree suggested autosomal dominant inheritance with reduced penetrance.

Patient III.1 (male, 55 years old) was hospitalized for syncope in 2005, and was recommended for permanent pacemaker implantation based on electrocardiography and the presence of a degree III atrioventricular block (24-h Holter examination). However, the patient rejected it. In 2016, the patient was admitted to the hospital, and reported recurrent symptoms that lasted for 10 years, chest tightness, and shortness of breath. The electrocardiography revealed a degree III block with atrial fibrillation, and a ventricular rate of 30–40 bpm. The patient underwent permanent pacemaker implantation. The echocardiography indicated a significantly increasing size in the left ventricular system.

Patient II.2 (female) underwent electrocardiography around 1979, which revealed a heart rate of 54 bpm, atrial fibrillation and a degree II atrioventricular block. The ultrasonic cardiography revealed left atrial enlargement, that is, the left atrial anterior and posterior diameters were 40 mm. The patient was admitted to our hospital with recurrent syncope, and line electrocardiography indicated atrial fibrillation, a degree III atrioventricular block, and a complete left bundle branch block. An attempt to implant a permanent pacemaker in 1982 was unsuccessful due to the abnormal vein structure. The patient was repeatedly admitted to our hospital over the subsequent four years due to chest tightness after activities, as well as shortness of breath. The electrocardiography revealed atrial fibrillation, a degree III atrioventricular block, and early polymorphism. The patient underwent permanent pacemaker implantation at another hospital in 1986.

Patients III.8 and III.11 exhibited different degrees of atrioventricular block. Patients II.2 and III.6 had syncope and atrial fibrillation, and underwent permanent pacemaker implantation. Patient II.6 had sinus bradycardia with a complete right bundle branch block, accompanied by atrial tachycardia and premature atrial beats. This patient did not receive a permanent pacemaker. Patient III.11 had a degree I atrioventricular block and sinus bradycardia, and had a history of acute myocardial infarction. This patient died of cardiogenic shock. Notably, the patient had a history of acute myocardial infarction. Hence, it was not inconceivable that this patient had ischemic cardiomyopathy, and the degree I atrioventricular block was very non-specific. Based on the medical history and clinical data available, it is not certain whether a degree I atrioventricular block occurred, regardless of the acute myocardial infarction, and it is not possible to determine whether the patient is a PCCD.

Among the members of the fourth generation who accepted gene test in this PCCD family (Table 9), the oldest one is 32 years old, the youngest one is 18 years old, members have no history of cardiovascular disease, electrocardiogram are sinus rhythm,has no arrhythmia, no heart palpitations, syncope and other clinical Symptoms, and no history of other diseases.

In summary, these results indicate that this family exhibited hereditary cardiac conduction disorder. In this family, no changes in myocardial structure or neurological abnormality were detected, and the muscle creatine kinase levels were within the normal range. Due to the symptomatic bradycardia, the proband received pacemaker implantation. Based on the II.2 recurrent syncope, this patient was diagnosed with atrial fibrillation associated with degree III atrioventricular block, and underwent permanent pacemaker implantation. Three cousins of the proband exhibited varying degrees of cardiac conduction disorder. Among these individuals, one woman (III.6) was implanted with a permanent pacemaker due to symptomatic bradycardia, and one male (III.3) was implanted with a permanent pacemaker due to a degree III atrioventricular block with atrial fibrillation and syncope symptoms. The other cousin did not show syncope or other symptoms, but had a degree I atrioventricular block, as detected by electrocardiography. Patients with syncope symptoms did not show convulsions in the syncope. Epilepsy was excluded due to the lack of epilepsy in the family history. Syncope caused by cerebrovascular disease was also excluded, because the head MRI and cervical vascular ultrasonography findings were normal. Furthermore, dynamic electrocardiography did not reveal frequent premature ventricle contraction or paroxysmal ventricular tachycardia, and no tachycardia was observed

Table 3
AAC variants among affected family members by WES.

Gene	Chr.	Pos.	Ref.	Alt.	Strand	Codon change	Amino acid substitution	Type
AHDC1	1	27,878,527	T	G	−1	CCC [A/C]CC CCC	T34P	Single AA change
ADAMTS4	1	161,166,384	G	A	−1	GAC C[C/T]T GAC	P307L	Single AA change
CHIT1	1	203,192,325	A	C	−1	CTG AG[T/G] GCA	S181R	Single AA change
MFF	2	228,220,473	G	A	1	AGA C[G/A]A CAG	R298Q	Single AA change
PTPN13	4	87,610,213	T	A	1	GAT G[T/A]T ATT	V139D	Single AA change
MANBA	4	103,644,047	T	C	−1	TGC C[A/G]T GTC	H177R	Single AA change
MUC2	11	1,092,928	T	A	1	ACA [T/A]CG ACA	S1583 T	Single AA change
CYB5R2	11	7,689,765	G	A	−1	CAG A[C/T]G AGT	T139 M	Single AA change
ANKRD11	16	89,347,729	C	T	−1	GCC [G/A]AC TCG	D1741N	Single AA change
PDYN	20	1,961,153	T	A	−1	GGG G[A/T]C GGG	D194V	Single AA change
PDYN	20	1,961,154	C	G	–1	GGG [G/C]AC GGG	D194H	Single AA change

AAC, amino acid coding (i.e. missense, nonsense, splice-site, indel); WES, whole exome sequencing.

before the syncope, excluding the possibility that the syncope was caused by malignant arrhythmia. Therefore, the syncope was attributed to the cardiac conduction disorder.

3.2. Sequencing analysis

Among the five living members of the family with PCCD, three members were available for whole-exome sequencing (III.1, III.3 and III.6; Table 3). The other two patients initially refused to undergo the sequencing (III.9 and III.11). Following the genetic testing and analysis of the family, it was confirmed that the absence of mutations in genes was known to cause PCCD (SCN5A, SCN1B, SCN10A, HCN4, KCNJ2, TRPM4, KCNK17, NKX2.5, TBX5, Cx40, PRKAG2, and LMNA).

Finally, a total of 11 point mutations in 10 genes were identified in three patients (Table 4) (The variants confirmed by WES in three affected family members was 37,452. The ACC variants that were absent from dbSNP and the 1000 Genomes Project was 418. A total of 11 point mutations in 10 genes were identified, which were heterozygous mutations). And gene nomenclature is according to HGVS. The bioinformatics analysis of the 10 genes using SIFT, Mutation Taster and PolyPhen-2 (Table 5) indicated that mutations in the *AHDC1* and *MUC2* genes were unlikely to be involved in the PCCD pathogenesis. PolyPhen-2 and SIFT predicts whether missense mutations cause changes in protein structure or function, while Mutation Taster predicts whether these mutations affect transcription or splicing. Sanger sequencing confirmed eight point mutations in seven genes in these three patients (Table 6), but it did not confirm the *CHIT1* mutation suggested by the whole-exome sequencing.

One PCCD patient in the family (III.8) ultimately consented to provide blood for Sanger sequencing. This revealed the presence of two point mutations in the *PDYN* gene (c.581A > T, c.580G > C; p.D194L), but there were no other mutations. These two point mutations were located in a conserved region of the propeptide (Fig. 1C–D).

Table 4
Filtering strategy for variants by whole-exome sequencing and Sanger sequencing.

	The number of variants
Total	37,452
The ACC variants that were absent from dbSNP and the 1000 Genomes Project	418
Total rare and novel by WES and database filtering Rare: present in ESP5400 with an MAF ≤ 0.4% Novel: absent from ESP5400	11
Bioinformatics analysis: using SIFT, Mutation Taster and PolyPhen-2 according to ACMG guideline	9
Confirmed by Sanger sequencing	8
Sanger sequencing analyzed mutations in other living patients (III.8): The same mutations in the patients members	2
SNaPshot sequencing for mutation validation	2

Two of three healthy members (III.9 and III.14) in the family donated their blood for Sanger sequencing (Table 7, Fig. 1B) and genotyping (Fig. 1A), and the results revealed a wild-type *PDYN* sequence in both. Unfortunately, subject III.16 and family members III.15, IV.9 and IV.10 refused to provide blood samples for the genetic tests.

The clinical analysis of 10 subjects in the fourth generation of the pedigree revealed that none of these subjects had any symptoms suggestive of PCCD. The findings of the 12-lead electrocardiography and ultrasonic cardiography were unremarkable. Subjects IV.1 and IV.2 carried two point mutations in the *PDYN* gene (c.581A > T, p.D194V and c. 580G > C, p.D194H) based on Sanger sequencing, while the other three subjects did not carry these mutations (Table 7, Fig. 1B). The remaining five subjects refused to provide blood samples for the genetic tests.

3.3. Sequencing validation using unrelated healthy control subjects

The SNaPShot, followed by the Sanger sequencing of 410 unrelated healthy control subjects, failed to reveal any mutation in the *PDYN* gene (Table 8).

4. Discussion

The major finding of the present study was that the two heterozygous mutations in *PDYN* (c.581A > T,c.580G > C; p. D194L) may constitute the pathogenic basis of PCCD in this family. PDYN is a precursor of Dyn in the opioid system. Mutations in *PDYN* are known to cause spinocerebellar ataxia (SCA) type 23 [19]. Cardiac autonomic dysfunction, which is predominantly in the parasympathetic system, has been noted in patients with SCA [20,21]. However, the individuals in the present pedigree exhibited no signs of SCA. That is, the neurology findings and muscle creatine kinase levels were normal.

In its early stages, PCCD usually shows no clinical manifestations and no abnormality on electrocardiography. The penetrance of the disease increases gradually with age [22,23]. Two individuals in the fourth generation of the present pedigree (IV.1, IV.2) were carriers of these pathogenic mutations, but had no symptoms, and had normal results on electrocardiography and ultrasonic cardiography (Table 9). The age of IV.1 and IV.2 are 18 and 27, respectively. In this family, patients with clinically observable PCCD had a normal heart structure, and no extracardiac disease phenotype.

Patients and families with PCCD, including families with readily observable genetic abnormalities, have been reported to present one or more of the following electrocardiographic phenotypes: atrioventricular block, sinus bradycardia, bundle branch block, and atrial arrhythmia. These presented in the form of atrial tachycardia, atrial fibrillation and premature atrial contraction [24,25]. PCCD has yet to be associated with congenital heart diseases, such as congenital

Table 5
Novel protein-altering variants ascertained by whole-exome sequencing in affected individuals (III.1, III.3, III.6).

Gene	cDNA change	Amino acid change	Chromosome	Position (hg19)	Polyphen-2 score[a]	Mutation taster[b]	SIFT score	GnoMAD score	CAAD score
AHDC1	c.100A > C	T34P	1	27,878,527	Benign (0)	N (0.995)	Tolerated (0.07)	–	14.29
ADAMTS4	c.920C > T	P307L	1	161,166,384	Possibly damaging (0.829)	Disease-causing (1)	Affects protein function (0)	–	25.5
CHIT1	c.543T > G	S181R	1	203,192,325	Probably damaging (0.986)	Disease-causing (0.619)	Affects protein function (0)	8.489e-5	13.48
MFF	c.893G > A	R298Q	2	228,220,473	Probably damaging (0.923)	Disease-causing (1)	Affects protein function (0)	1.198e-5	37
PTPN13	c.416T > A	V139D	4	87,610,213	Possibly damaging (0.883)	Disease-causing (1)	Affects protein function (0.04)	4.014e-6	22.9
MANBA	c.530A > G	H177R	4	103,644,047	Possibly damaging (0.739)	Disease-causing (1)	Affects protein function (0)	2.652e-4	11.18
MUC2	c.4747T > A	S1583 T	11	1,092,928	Benign (0)	N (1)	Tolerated (0.61)	–	0.629
CYB5R2	c.416C > T	T139 M	11	7,689,765	Benign (0.018)	N (1)	Affects protein function (0)	2.784e-5	11.78
ANKRD11	c.5221G > A	D1741N	16	89,347,729	Possibly damaging (0.818)	Disease-causing (1)	Affects protein function (0.01)	–	19.41
PDYN	c.581A > T	D194V	20	1,961,153	Benign (0.009)	Disease-causing (1)	Affects protein function (0.01)	3.979e-5	8.519
PDYN	c.580G > C	D194H	20	1,961,154	Possibly damaging (0.533)	Disease-causing (0.994)	Affects protein function (0.02)	2.476e-5	15.11

Note: SIFT, scale-invariant feature transform.
[a] Lower scores indicate more benign substitutions.
[b] Probability that the prediction is accurate.

Table 6
Sanger sequencing to verify nine candidate mutations.

Gene	cDNA change	Sanger sequencing results for subject		
		III.1	III.3	III.6
ADAMTS4	c.920C > T	CT	CT	CT
CHIT1	c.543T > G	T	T	T
MFF	c.893G > A	GA	GA	GA
PTPN13	c.416T > A	TA	TA	TA
MANBA	c.530A > G	AG	AG	AG
CYB5R2	c.416C > T	CT	CT	CT
ANKRD11	c.5221G > A	AG	AG	AG
PDYN	c.581A > T	AT	AT	AT
PDYN	c.580G > C	GC	GC	GC

Table 7
PDYN mutations detected by Sanger sequencing within the pedigree.

Pedigree	c.581A > T; p.D194V	c.580G > C; p.D194H
III.1	AT	CG
III.2	A	G
III.3	AT	CG
III.4	A	G
III.6	AT	CG
III.8	AT	CG
III.9	A	G
III.11	AT	CG
III.12	A	G
III.14	A	G
IV.1	AT	CG
IV.2	AT	CG
IV.6	A	G
IV.7	A	G
IV.8	A	G

Table 8
SNaPshot results of *PDYN* mutations in 410 healthy individuals.

Pathogenic *PDYN* mutation	Genotype (n)		
c.581A > T	AA (410)	AT (0)	TT (0)
c.580G > C	GG (410)	GC (0)	CC (0)

Table 9
Clinical characteristics of the IV.1 and IV.2 in this PCCD family.

Clinical characteristics			Echocardiography		
ID	Sex	Age	LVEF (%)	LA (mm)	LV (mm)
IV.1	M	18	61	33	47
IV.2	F	27	61	30	45
IV.6	F	32	60	26	40
IV.7	M	18	62	37	50
IV.8	F	26	61	30	45

Note: F, female; ID, subject index; LA, left atrium; LV, left ventricle; LVEF, left ventricular ejection fraction; M, male.

atrioventricular block or atrial septal defects [26]. Similarly, it has yet to be associated with cardiomyopathy or other phenotypes that involve extracardiac organs and tissues, such as skeletal muscle myopathy [27].

Patient II.2 in the present study had symptoms, and was diagnosed earliest with atrioventricular block. This patient received a conduction diagnosis at the age of 39 years old. PCCD usually progresses with age. Young members of affected families may have normal electrocardiography results, but may continue to be at risk of arrhythmia, and the risk of sudden death increases with age. The genetic testing of hereditary disease is crucial in identifying asymptotic individuals with

the same mutation(s) as symptomatic family members. Patients with negative genetic test results can be assured that they are unlikely to develop PCCD.

The present results establish a link between the opioid system and PCCD. In addition to these analgesic properties, opioids induce arterial hypotension and bradycardia. These effects appear to be transmitted via the dorsal vagal complex in the central nervous system [28]. The activation of cardiac opioid receptors can protect against myocardial infarction, and may trigger processes similar to ischemic preconditioning [29]. The stimulation of cardiac KOR activates a subcellular kinase pathway, in which the opening of sarcolemmal and mitochondrial ATP-sensitive K^+-channels (K^+_{ATP}) appear to protect against ischemia-induced calcium overload [30,31]. KOR ligands inhibit isoprenaline-induced cardiachypertrophy [32], and prevent increases in lipid peroxidation and the depletion of myocardial antioxidants [33], thereby reducing oxidative stress. Sascha et al. [34] found that the opioid receptor is associated with a myocardial excitatory-contractile coupling mechanism and oxidative phosphorylation in redox reactions. It was speculated that reactive oxygen species may inactivate Ca^{2+}-ATPase on the sarcoplasmatic reticulum, reducing its ability to take in and release Ca^{2+}. This may affect the action potential of cardiomyocytes, and cause cardiac conduction disorders.

The present results may help guide studies to examine how opioids affect electrophysiology in cardiac conduction. Although some case reports have suggested that opioids exert direct effects on conduction, substantial evidence exists that opioids affect myocytes. In the heart, the opioid system is an important regulator of β-adrenergic receptor signal transduction, excitatory-contractile coupling, and cardiac development [35,36]. Interestingly, five of six PCCD patients in the family had atrial arrhythmias at the same time, while three patients had atrial fibrillation. It was speculated that atrial fibrillation is a key clinical indicator of the interaction between endogenous opioids and cardiac conduction. One study found that the levels of opioid peptide precursors and KOR mRNA are lower in patients with atrial fibrillation than in a sinus rhythm group [37], while the REGARDS study found that opioid drug use is independently associated with the increased prevalence of atrial fibrillation [38]. Endogenous opioid peptides open mitochondrial K^+_{ATP} channels, rendering mitochondria resistant to oxidative stress during ischemia. The loss of this protective mechanism may render atrial myocytes susceptible to damage, and may lead to atrial fibrillation [39]. Therefore, the investigators propose a correlation between endogenous opioid peptide levels and atrial fibrillation, and suspect that atrial arrhythmia in most of the present pedigree patients was associated with KORs. Future work is needed to clarify whether changes in opioid receptor expression affect arrhythmia.

5. Conclusion

The present study reports the first evidence that a *PDYN* mutation (c.581A > T,c.580G > C; p. D194L) is specifically associated with PCCD. The present findings show that PCCD is associated with the opioid system. *PDYN* mutations may affect the cardiac conduction system by activating the KOR to affect the Ca^{2+}-ATPaseor K^+_{ATP} channels. Further studies are needed to elucidate the mechanism by which mutant PDYN acts on the cardiac conduction system.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

References

[1] A.E. Baruteau, V. Probst, H. Abriel, Inherited progressive cardiac conduction disorders, Curr. Opin. Cardiol. 30 (1) (2015) 33–39.

[2] E. Gilbert-Barness, Conduction defects/cardiomyopathies, Adv. Pediatr. Infect. Dis. 61 (1) (2014) 127–148.

[3] S.B. Ng, E.H. Turner, P.D. Robertson, S.D. Flygare, A.W. Bigham, C. Lee, T. Shaffer, M. Wong, A. Bhattacharjee, E.E. Eichler, M. Bamshad, D.A. Nickerson, J. Shendure, Targeted capture and massively parallel sequencing of 12 human exomes, Nature 461 (7261) (2009) 272–276.

[4] M. Choi, U.I. Scholl, W. Ji, T. Liu, I.R. Tikhonova, P. Zumbo, A. Nayir, A. Bakkaloğlu, S. Ozen, S. Sanjad, C. Nelson-Williams, A. Farhi, S. Mane, R.P. Lifton, Genetic diagnosis by whole exome capture and massively parallel DNA sequencing, Proc. Natl. Acad. Sci. U. S. A. 106 (45) (2009) 19096–19101.

[5] M.J. Clark, R. Chen, H.Y. Lam, K.J. Karczewski, R. Chen, G. Euskirchen, A.J. Butte, M. Snyder, Performance comparison of exome DNA sequencing technologies, Nat. Biotechnol. 29 (10) (2011) 908–914.

[6] K. Tsou, C.S. Jang, Studies on the site of analgesic action of morphine by intracerebral microinjection, Sci. Sinica (1964) 1099–1109.

[7] J.L. Caffrey, M.O. Boluyt, A. Younes, B.A. Barron, L. O'Neill, M.T. Crow, E.G. Lakatta, Aging, cardiac proenkephalin mRNA and enkephalin peptides in the Fisher 344 rat, J. Mol. Cell. Cardiol. 26 (6) (1994) 701–711.

[8] L.E. Miller, G.R. McGinnis, B.A. Peters, C.G. Ballmann, G. Nanayakkara, R. Amin, J.C. Quindry, Involvement of the δ-opioid receptor in exercise-induced cardioprotection, Exp. Physiol. 1;100 (4) (2015) 410–421.

[9] R.D. Howells, D.L. Kilpatrick, L.C. Bailey, M. Noe, S. Udenfriend, Proenkephalin mRNA in rat heart, Proc. Natl. Acad. Sci. U. S. A. 83 (6) (1986) 1960–1963.

[10] S. Treskatsch, A. Feldheiser, M. Shaqura, L. Dehe, H. Habazettl, T.K. Röpke, M. Shakibaei, M. Schäfer, C.D. Spies, S.A. Mousa, Cellular localization and adaptive changes of the cardiac delta opioid receptor system in an experimental model of heart failure in rats, Heart Vessel. 31 (2) (2016) 241–250.

[11] D.C. Siong Chan, T.H. Cao, L.L. Ng, Proenkephalin in heart failure, Heart Fail. Clin. 14 (1) (2018) 1–11.

[12] M.A. Romano, E.M. Seymour, J.A. Berry, R.A. McNish, S.F. Bolling, Relative contribution of endogenous opioids to myocardial ischemic tolerance, J. Surg. Res. 118 (1) (2004) 32–37.

[13] O. Oldenburg, M.V. Cohen, D.M. Yellon, J.M. Downey, Mitochondrial K(ATP) channels: role in cardioprotection, Cardiovasc. Res. 55 (3) (2002) 429–437.

[14] M.N. Foster, W.A. Coetzee, KATP channels in the cardiovascular system, Physiol. Rev. 96 (1) (2016) 177–252.

[15] T.M. Wong, A.Y. Lee, K.K. Tai, Effects of drugs interacting with opioid receptors during normal perfusion or ischemia and reperfusion in the isolated rat heart–an attempt to identify cardiac opioid receptor subtype(s) involved in arrhythmogenesis, J. Mol. Cell. Cardiol. 22 (1990) 1167–1175.

[16] M.J. Ackerman, S.G. Priori, S. Willems, C. Berul, R. Brugada, H. Calkins, A.J. Camm, P.T. Ellinor, M. Gollob, R. Hamilton, R.E. Hershberger, D.P. Judge, H. Le Marec, W.J. McKenna, E. Schulze-Bahr, C. Semsarian, J.A. Towbin, H. Watkins, A. Wilde, C. Wolpert, D.P. Zipes, Heart Rhythm Society (HRS), European Heart Rhythm Association (EHRA), HRS/EHRA expert consensus statement on the state of genetic testing for the channelopathies and cardiomyopathies: this document was developed as a partnership between the Heart Rhythm Society (HRS) and the European Heart Rhythm Association (EHRA), Europace 13 (8) (2011) 1077–1109.

[17] S.G. Priori, C. Blomstrom-Lundqvist, 2015 European Society of Cardiology Guidelines for the management of patients with ventricular arrhythmias and the prevention of sudden cardiac death summarized by co-chairs, Eur. Heart J. 36 (41) (2015) 2757–2759.

[18] S.G. Priori, A.A. Wilde, M. Horie, Y. Cho, E.R. Behr, C. Berul, N. Blom, J. Brugada, C.E. Chiang, H. Huikuri, P. Kannankeril, A. Krahn, A. Leenhardt, A. Moss, P.J. Schwartz, W. Shimizu, G. Tomaselli, C. Tracy, HRS/EHRA/APHRS expert consensus statement on the diagnosis and management of patients with inherited primary arrhythmia syndromes: document endorsed by HRS, EHRA, and APHRS in May 2013 and by ACCF, AHA, PACES, and AEPC in June 2013, Heart Rhythm. 10 (12) (2013) 1932–1963.

[19] G. Bakalkin, H. Watanabe, J. Jezierska, C. Depoorter, C. Verschuuren-Bemelmans, I. Bazov, K.A. Artemenko, T. Yakovleva, D. Dooijes, B.P. Van de Warrenburg, R.A. Zubarev, B. Kremer, P.E. Knapp, K.F. Hauser, C. Wijmenga, F. Nyberg, R.J. Sinke, D.S. Verbeek, Prodynorphin mutations cause the neurodegenerative disorder spinocerebellar ataxia type 23, Am. J. Hum. Genet. 87 (5) (2010) 593–603.

[20] J. Montes-Brown, G. Sanchez-Cruz, A.M. Garcia, M.E. Baez, L. Velazquez-Perez, Heart rate variability in type 2 spinocerebellar ataxia, Acta Neurol. Scand. 122 (5) (2010) 329–335.

[21] C. Pradhan, B.S. Yashavantha, P.K. Pal, T.N. Sathyaprabha, Spinocerebellar ataxias type 1, 2 and 3: a study of heart rate variability, Acta Neurol. Scand. 117 (5) (2008) 337–342.

[22] V. Probst, F. Kyndt, F. Potet, J.N. Trochu, G. Mialet, S. Demolombe, J.J. Schott, I. Baró, D. Escande, H. Le Marec, Haploinsufficiency in combination with aging causes SCN5A-linked hereditary Lenegre disease, J. Am. Coll. Cardiol. 41 (4) (2003) 643–652.

[23] C.A. Martin, C.L. Huang, A.A. Grace, Progressive conduction diseases, Card. Electrophysiol. Clin. 2 (4) (2010) 509–519.

[24] P.G. Postema, M. Van den Berg, J.P. Van Tintelen, F. Van den Heuvel, M. Grundeken, N. Hofman, W.P. Van der Roest, E.A. Nannenberg, I.P. Krapels, C.R. Bezzina, A. Wilde, Founder mutations in the Netherlands: SCN5a 1795insD, the first described arrhythmia overlap syndrome and one of the largest and best characterised families worldwide, Neth. Hear. J. 17 (11) (2009) 422–428.

[25] D.W. Benson, D.W. Wang, M. Dyment, T.K. Knilans, F.A. Fish, M.J. Strieper, T.H. Rhodes, A.L. George Jr., Congenital sick sinus syndrome caused by recessive mutations in the cardiac sodium channel gene (SCN5A), J. Clin. Invest. 112 (7) (2003) 1019–1028.

[26] J.J. Schott, D.W. Benson, C.T. Basson, W. Pease, G.M. Silberbach, J.P. Moak, B.J. Maron, C.E. Seidman, J.G. Seidman, Congenital heart disease caused by mutations in the transcription factor NKX2-5, Science 281 (5373) (1998) 108–111.

[27] M. Paul, S. Zumhagen, B. Stallmeyer, M. Koopmann, T. Spieker, E. Schulze-Bahr, Genes causing inherited forms of cardiomyopathies. A current compendium, Herz 34 (2) (2009) 98–109.
[28] P.D. Feldman, N. Parveen, S. Sezen, Cardiovascular effects of Leu-enkephalin in the nucleus tractus solitarius of the rat, Brain Res. 709 (2) (1996) 331–336.
[29] J.E. Schultz, G.J. Gross, Opioids and cardioprotection, Pharmacol. Ther. 89 (2) (2001) 123–137.
[30] Z. Cao, L. Liu, D.M. Van Winkle, Activation of delta- and kappa-opioid receptors by opioid peptides protects cardiomyocytes via KATP channels, Am. J. Physiol. Heart Circ. Physiol. 285 (3) (2003) H1032–H1039.
[31] M. Chen, J.J. Zhou, K.W. Kam, J.S. Qi, W.Y. Yan, S. Wu, T.M. Wong, Roles of KATP channels in delayed cardioprotection and intracellular Ca(2+) in the rat heart as revealed by kappa-opioid receptor stimulation with U50488H, Br. J. Pharmacol. 140 (4) (2003) 750–758.
[32] W. Yin, P. Zhang, J.H. Huang, Q.Y. Zhang, R. Fan, J. Li, J.J. Zhou, Y.Z. Hu, H.T. Guo, S.M. Zhang, Y.M. Wang, A.D. Kaye, C.H. Gu, J.C. Liu, L. Cheng, Q. Cui, D.H. Yi, J.M. Pei, Stimulation of kappa-opioid receptor reduces isoprenaline-induced cardiac hypertrophy and fibrosis, Eur. J. Pharmacol. 607 (1–3) (2009) 135–142.
[33] A. Jaiswal, S. Kumar, S. Seth, A.K. Dinda, S.K. Maulik, Effect of U50,488H, a kappa-opioid receptor agonist on myocardial alpha-and beta-myosin heavy chain expression and oxidative stress associated with isoproterenol-induced cardiac hypertrophy in rat, Mol. Cell. Biochem. 345 (1–2) (2010) 231–240.
[34] S. Treskatsch, M. Shaqura, L. Dehe, A. Feldheiser, T.K. Roepke, M. Shakibaei, C.D. Spies, M. Schäfer, S.A. Mousa, Upregulation of the kappa opioidergic system in left ventricular rat myocardium in response to volume overload: adaptive changes of the cardiac kappa opioid system in heart failure, Pharmacol. Res. 102 (2015) 33–41.
[35] M.K. Pugsley, The diverse molecular mechanisms responsible for the actions of opioids on the cardiovascular system, Pharmacol. Ther. 93 (1) (2002) 51–75.
[36] J.P. Headrick, S. Pepe, J.N. Peart, Non-analgesic effects of opioids: cardiovascular effects of opioids and their receptor systems, Curr. Pharm. Des. 18 (37) (2012) 6090–6100.
[37] U. Lendeckel, C. Muller, C. Rocken, B. Laube, M. Tager, C. Huth, H.U. Klein, A. Goette, Expression of opioid receptor subtypes and their ligands in fibrillating human atria, Pacing Clin. Electrophysiol. 28 (Suppl. 1) (2005) S275–S279.
[38] W.T. Qureshi, W.T. O'Neal, Y. Khodneva, S. Judd, M.M. Safford, P. Muntner, E.Z. Soliman, Association between opioid use and atrial fibrillation: the Reasons for Geographic and Racial Differences in Stroke (REGARDS) study, JAMA Intern. Med. 175 (6) (2015) 1058–1060.
[39] E.L. Holmuhamedov, L. Wang, A. Terzic, ATP-sensitive K+ channel openers prevent Ca2+ overload in rat cardiac mitochondria, J. Physiol. 519 (Pt 2) (1999) 347–360.