Contactin-2 Expression in the Cardiac Purkinje Fiber Network

Benedetta A. Pallante, PhD, DVM; Steven Giovannone, MD; Liu Fang-Yu; Jie Zhang; Nian Liu, MD; Guoxin Kang, PhD; Wen Dun, PhD; Penelope A. Boyden, PhD; Glenn I. Fishman, MD

Background—Purkinje cells (PCs) comprise the most distal component of the cardiac conduction system, and their unique electrophysiological properties and the anatomic complexity of the Purkinje fiber network may account for the prominent role these cells play in the genesis of various arrhythmic syndromes.

Methods and Results—Differential transcriptional profiling of murine Purkinje fibers and working ventricular myocytes was performed to identify novel genes expressed in PCs. The most highly enriched transcript in Purkinje fibers encoded Contactin-2 (Cntn2), a cell adhesion molecule critical for neuronal patterning and ion channel clustering. Endogenous expression of Cntn2 in the murine ventricle was restricted to a subendocardial network of myocytes that also express β-galactosidase in CCS-lacZ transgenic mice and the connexin40 gap junction protein. Both Cntn2-lacZ knockin mice and Cntn2-EGFP BAC transgenic reporter mice confirmed expression of Cntn2 in the Purkinje fiber network, as did immunohistochemical staining of single canine Purkinje fibers. Whole-cell patch-clamp recordings and measurements of Ca\(^{2+}\) transients in Cntn2-EGFP\(^{+}\) cells revealed electrophysiological properties indicative of PCs and distinctive from those of cardiac myocytes, including prolonged action potentials and frequent afterdepolarizations.

Conclusions—Cntn2 is a novel marker of the specialized cardiac conduction system. Endogenous expression of Cntn2 as well as Cntn2-dependent transcriptional reporters provides a new tool through which Purkinje cell biology and pathophysiology can now more readily be deciphered. Expression of a contactin family member within the CCS may provide a mechanistic basis for patterning of the conduction system network and the organization of ion channels within Purkinje cells. (Circ Arrhythm Electrophysiol. 2010;3:186-194.)

Key Words: cell adhesion molecules | electrophysiology | genetics | Purkinje fiber

Purkinje fibers (PFs) are the most distal component of the cardiac conduction system (CCS), first described by Purkinje in 1839 as gray, flat, gelatin-like ramifications, running under the endocardium. Some 70 years later, Tawara more fully characterized the Purkinje system, identifying the left (anterior, septal, and posterior) and right fascicular strands, which served to connect the distal PFs to the bundle branches proper. Tawara was also the first to correctly suggest the functional role of the Purkinje system in rapidly transmitting the electric wave of excitation to the ventricular muscle.

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PFs appear to play a prominent role in the genesis of ventricular arrhythmias, (reviewed in Reference 3). PF and anterior or posterior left fascicular triggers have been implicated in the initiation of monomorphic ventricular tachycardia in post–myocardial infarction patients, as demonstrated by cure after focal ablation of Purkinje fiber or fascicular potentials.4–6 PF-based triggers have also been described in patients with ventricular tachycardia associated with dilated forms of cardiomyopathy,7 as well as idiopathic ventricular fibrillation (VF), in which ablation of premature beats arising from the PF network resulted in significant reductions in the recurrence of VF.8 PF-dependent triggering of arrhythmias has also been proposed in inherited syndromes including catecholaminergic polymorphic VT,9 Brugada syndrome, and long-QT syndrome.10

Despite growing evidence implicating PFs in ventricular arrhythmogenesis, our understanding of the cellular mechanisms underlying PF-dependent diseases is hampered by the lack of knowledge of the developmental biology of individual Purkinje cells (PCs), their patterning into a network of highly coupled cells, and their adaptive and maladaptive responses to pathological stimuli. To some extent, this gap in knowledge reflects the anatomic complexity of the PF network, which includes branching cells that couple not only with neighboring PCs but also with working myocytes at Purkinje-
muscle junctions. Furthermore, PFs are thought to include cells with functional and structural heterogeneity, such as the transitional J-cells.11

In recent years, a number of “conduction-system” markers have been reported.12–14 These include transcription factors, gap junction–associated proteins, and ion channel–associated proteins, with different specificities and different roles in the development and function of the CCS. However, the majority of these molecular markers, including HF-1b15 and Kcne1,16 preferentially identify cells of the proximal CCS, and, to date, only 2, the CCS-lacZ gene (resulting from insertion of an Engrailed-2 reporter gene into the Slco3a1 locus17,18) and gju5/connexin40 (Cx40), are robustly expressed in PFs.

In the developing mouse ventricle, CCS-lacZ expression is first observed in trabecular myocardium at embryonic day 10.5, a domain that appears to be the primary source of cells contributing to the developing PF network. By E13.5, the reporter gene expression pattern is similar to the neonate, with the appearance of a more discrete subendocardial fiber network.19 Expression is also observed in other components of the CCS, including both the sinoatrial node (SAN) and atroventricular node (AVN), as well as the His bundle and bundle branches.20 Unfortunately, expression of β-galactosidase in the CCS-lacZ strain is the result of complex insertional mutation associated with substantial genomic rearrangement, complicating mechanistic studies of CCS-specific transcriptional regulation.18

Cx40 is a gap junction protein expressed in the developing and adult murine heart,21 and Cx40-EGFP reporter mice have also been used to visualize the ventricular conduction system.22 Cx40 expression is observed in other components of the CCS (AVN, His bundle and bundle branches) but is also abundantly expressed in atrial myocytes and coronary arteries. Examination of Cx40 deficient murine models has provided insight into its important role in development and function of the CCS. Loss of function of Cx40 results in an increased incidence of cardiac malformations (double-outlet right ventricle, dilated and hypertrophic cardiomyopathy)23 as well as conduction defects.24 Interestingly, sequence variants within the human GJA5 locus have been described in association with tetralogy of Fallot.25

There are also reports12,14 describing PF-enriched expression of 2 cardiac homeobox transcription factors: Nkx2-5 and Homeodomain only protein (Hop). Nkx2-5−/− mice are characterized by ventricular conduction defects specifically attributable to a hypoplastic PF network, thought to be the result of impaired postnatal differentiation of myocytes.12 However, Nkx2-5 is broadly expressed in many cell types within the developing and adult heart, which diminishes its utility as a PF-specific marker. Hop is a cardiac homeobox transcription factor that acts downstream of Nkx2-5.14,26 Hop-lacZ knockin mice show broad reporter gene expression in the developing heart, which becomes somewhat more restricted to the atria and proximal CCS in the adult. Accordingly, with the goal of developing additional specific markers of the distal CCS that might be useful to facilitate studies elucidating the molecular circuitry regulat-

ing CCS specification, patterning, and function, we performed transcriptional profiling of the murine PF network.

Methods

Methods are described in brief here (see the online-only Data Supplement for a full description).

Mutant Mice

All experiments were performed according to protocols approved by the NYU Institutional Animal Care and Use Committee and conformed to the National Institutes of Health (NIH) guidelines for the care and use of Laboratory Animals. CCS-lacZ−/− transgenic (back-crossed into the CD-1 background for at least 9 generations)19 and Cntn2-lacZ knockin mice (on a C57BL/6x129SvEv mixed background)27 have been previously described. Cntn2-EGFP BAC transgenic mice [Tg(Cntn2-EGFP)344sat] were obtained from MMRR and maintained on a CD-1 background.

Microarray Profiling

PF-enriched samples were obtained by microdissecting the subendocardial layer of 6- to 8-month-old CCS-lacZ murine hearts, and PF-depleted samples were microdissected from the subepicardial layer of the ventricular walls.

One-Step Reverse Transcription and Polymerase Chain Reaction and Quantitative Real-Time Reverse Transcription and Polymerase Chain Reaction

Total RNA was processed using the Quantitect SYBR Green One-Step reverse transcription and polymerase chain reaction (RT-PCR) kit (Qiagen) or One-Step SuperScript III (Invitrogen). In quantitative real-time PCR (QRT-PCR) experiments, quantification of transcripts was performed using GAPDH as an internal reference and the 2−ΔΔCT method.28 For gene specific primers, see Data Supplement.

Isolation of Adult Canine Purkinje Fibers and Individual Purkinje Cells

PCs were enzymatically dispersed from PFs of the canine heart, as previously described.29,30

Immunostaining

Mouse hearts or canine PFs were fixed in 4% PFA, then equilibrated in 10% sucrose and embedded in OCT. Cryosections 6 to 10 μm thick were processed for staining with individual antibodies as detailed in the Data Supplement.

Electrophysiological Recordings in Isolated Ventricular Myocytes

Ventricular myocytes were isolated using an established enzymatic digestion protocol yielding to 60% to 80% rod-shaped, calcium-tolerant myocytes. Action potential duration (APD) was measured at 90% and 50% of repolarization (APD90 and APD50). Delayed afterdepolarizations (DADs) were defined as phase 4 positive (depolarizing) deflections of the membrane potential. Early afterdepolarizations (EADs) were defined as positive (depolarizing) oscillations occurring during phase 2 or 3 of action potential.

Ca2+ Imaging

Isolated myocytes from Cntn2-EGFP transgenic mice were loaded with the membrane-permeant Ca2+ indicator dye Rhod-2/AM and imaged by confocal microscopy (Leica SP5). In some experiments, the Ca2+ transients were measured by microfluorimetry (IonOptix, Milton, Mass) using Indo-1/AM.
Statistics
Data are presented as mean±SEM. Values were compared with a 2-tailed t test, and probability values <0.05 were considered statistically significant.

Results
Cntn2 Transcripts Are Preferentially Expressed in Cardiac PFs
Using CCS-lacZ hearts, in which the distal conduction system is readily visualized, we performed comparative transcriptional profiling of mRNA prepared from PF-enriched subendocardial fractions harvested distal to the bundle branches proper and from PF-deficient subepicardial layers (Figure 1A). Enrichment of PFs was confirmed by X-Gal staining of dissected tissue (Figure 1B, top) and by RT-PCR, which showed preferential expression of transcripts encoding both β-galactosidase as well as connexin40 (Figure 1B, bottom).

Comparison of global gene expression patterns from 3 independent datasets of PF-enriched and PF-depleted samples by microarray analysis (Dataset Supplement) identified 163 probe sets that were significantly upregulated (5.87- to 1.33-fold increase) and 251 that were downregulated (0.77- to 0.26-fold decrease). The transcript encoding Contactin2

Figure 1. Identification of novel PF-specific transcripts by comparative microarray analysis. A, PF- and PF+ samples were microdissected distal to the bundle branches (top, arrow) from the subendocardial and subepicardial regions (bottom) of CCS-lacZ hearts. B, Enrichment of PF-specific transcripts in PF+ samples was confirmed by increased expression of the PF markers CCS-lacZ and Cx40 compared with PF- samples, assessed by X-Gal staining (top) and/or RT-PCR (bottom). C, QRT-PCR showing upregulation of Cntn2 and the PF-specific markers CCS-lacZ (β-Gal) and Cx40 in microdissected PFs. Values are expressed as arbitrary units (1 AU=RV in PF- sample). RW indicates right ventricular wall; LW, left ventricular wall; RV, right ventricle; LV, left ventricle; e, endocardium; E, epicardium. Black outline defines subendocardial region; red outline, subepicardial region; IS, interventricular septum. Size bar: 200 μM.

Figure 2. Cntn2 is specifically expressed in PFs. A (left), Confocal image of mouse heart sections confirming that Cntn2− cells (green) express the cardiac marker sarcomeric actinin (red, left). Inset: High-power image showing an optical slice of a Cntn2−/sarcomeric actinin− myocyte. B (left), Epifluorescent images of CCS-lacZ hearts showing a Cntn2+βGal− (green/red) cell (arrowhead) under a layer of Cntn2−βGal− endocardial cells (arrows). B (right), Images of CCS-lacZ heart serial sections stained for X-Gal (upper) and Cntn2 (red, lower) showing colocalization of Cntn2 and the PF-specific marker CCS-lacZ. C, Epifluorescent image of E13.5 mouse brain used as positive control, showing Cntn2 expression (red) in the intermediate and marginal zones (arrowheads) and in the preplate region (arrows) of the ventral telenencephalon. LW indicates, left ventricular wall; LV, left ventricle; RW, right ventricular wall; RV, right ventricle. Size bars: 20 μM. A (left, inset), 35 μM.
ingly, Sema3b (Figure 1C). Of note, 2 members of the Iroquois gene family, (left, X-Gal staining) and /H11001 the walls (conduction system disease.32 SCN10A the PF fraction (2.15-fold). Sequence variation in the TTX-resistant sodium channel isoform, was also enriched in

C. Size bars: A, 50 μM, all except for bottom, left, 500 μM; B, 20 μM, all except for top, left, 200 μM, and bottom, left, 50 μM; C, 100 μM.

Figure 3. Cntn2 expression delineates the CCS in the adult heart. A, Whole mounts (top) and cryosections (bottom) of adult Cntn2-lacZ (left, X-Gal staining) and Cntn2-EGFP hearts (Right) showing expression of Cntn2 throughout the CCS. B, Epifluorescent images of Cntn2-EGFP hearts confirming expression of Cntn2 (green) in the SAN (top), AVN (center), and PFs (bottom), identified by anatomic landmarks and expression of the nodal marker Hcn4 (red) or the PF marker Cx40 (red). Top: Cntn2-EGFP+ cells (green) were found in the SAN, localized at the junction of the VC with the right atrium and extending from the CT (0 μM) posterior to the base (+40 μM) and the walls (+190 μM) of the VC anteriorly. Cntn2 identifies a subpopulation of elongated Hcn4+ cells in the SAN. Cntn2-EGFP+/Hcn4+ cells were also found in the AVN, 20 to 40 μM posterior to the His bundle (center). Bottom: Cntn2-EGFP+ (green)/Cx40+ (red) PFs were observed in both ventricles. C, Cntn2-EGFP transgene expression recapitulates Cntn2 protein expression as shown by colocalization of anti-Cntn2-TR antibody (red) and EGFP (green) signals in the AVN (top) and the PFs (bottom). Bottom right: Confocal image detail of PFs. His/Br indicates His bundle and bundle branches; rPFs, right ventricle PFs; Ct, crista terminalis; VC, vena cava; A, aorta and pulmonary artery; arrow, SAN (in B). Blue fluorescence: DAPI nuclear counter stain, in A (bottom, right), B, and C. Size bars: A, 50 μM, all except for bottom, left, 500 μM; B, 20 μM, all except for top, left, 200 μM, and bottom, left, 50 μM; C, 100 μM.

(Cntn2; UniGene Mm.34775), showed the highest level of enrichment (5.87). RT-PCR was used to confirm the microarray results identifying Cntn2 expression in cardiac PFs (Figure 1C). Of note, 2 members of the Iroquois gene family, Irx5 (4.18) and Irx2 (2.89), were also enriched in PFs, as was Sema3b, a putative molecular partner of contactins. Interestingly, Nav1.8/SCN10A, which encodes the α-subunit of a TTX-resistant sodium channel isoform, was also enriched in the PF fraction (2.15-fold). Sequence variation in the SCN10A gene has recently been associated with human conduction system disease.32

Cntn2 Is Expressed on the Sarcolemma of Murine Purkinje Cells
We performed immunohistochemical staining to specifically localize expression of Cntn2 in the heart. Cryosections of adult murine hearts were costained for Cntn2 as well as the cardiac-specific marker sarcomeric actinin, which revealed that Cntn2+ cells belong to the cardiac lineage (Figure 2A, left). Costaining of CCS-lacZ hearts for Cntn2 and β-galactosidase (β-Gal) or staining of serial sections for Cntn2 or X-Gal, confirmed that Cntn2+ cells were also βGal+ (Figure 2B, left) or X-Gal+ (Figure 2B, right). Furthermore, these images also demonstrated that the Cntn2-positive cells were localized just beneath the thin endocardial cell layer (Figure 2B, left), similar to what we previously reported using the CCS-lacZ marker.20 Taken together, these data indicate that Cntn2 is specifically expressed in adult murine PFs.

Cntn2 Is Expressed in the Proximal and Distal CCS
To more fully examine the spatial pattern of Cntn2 in the adult murine heart, we used 2 independent reporter gene mice: Cntn2-lacZ knockin mice and Cntn2-EGFP BAC transgenic mice. Examination of the endocardial surfaces of the ventricular cavities of whole mount Cntn2-lacZ hearts (Figure 3A, top, left) and Cntn2-EGFP (Figure 3A, top, right) hearts revealed dense networks of X-Gal+ and EGFP+ fluorescent PFs, which were also observed after sectioning (Figure 3A, bottom). Detailed histological examination of Cntn2-EGFP hearts also demonstrated expression in cells of the sinoatrial node (SAN; Figure 3B, top),13 including cells close to the crista terminalis in the posterior part of the SAN; at the base of the vena cava in the central part of the SAN; and in the walls of the vena cava in the most anterior part of the SAN. Coexpression of Cntn2 and the nodal marker Hcn4 further confirmed that Cntn2 is indeed expressed in a subpopulation of elongated Hcn4+ cells in the SAN. Detailed examination of the His bundle/AVN region revealed expression of Cntn2-EGFP in Hcn4+ cells of the AVN, localized
posterior to the His bundle (Figure 3B, center). Costaining for connexin40 (Cx40) also confirmed expression of Cntn2-EGFP in Cx40+/H11001 PCs of both ventricles (Figure 3B, bottom). Staining with anti-Cntn2 antibodies confirmed that EGFP reporter gene expression in Cntn2-EGFP transgenic mice correctly reflected endogenous gene expression, as shown by colocalization of the 2 markers in components of the CCS (Figure 3C).

**Cntn2 Expression in Canine PFs**

Immunohistochemistry of isolated canine PFs showed preferential expression of Cntn2 on the sarcolemma of individual PCs (Figure 4A). Myocytes expressing Cntn2 on their cell membrane also expressed the PC marker Cx40 at their intercalated disks, as observed in both longitudinal (Figure 4B, left) and tangential (Figure 4B, right) cell sections. Confocal images of dissociated canine PCs (Figure 4C) confirmed the expression of Cntn2 on the sarcolemma of Cx40+ cells with a rod-shaped, Purkinje-like phenotype. The cardiac pore-forming sodium channel subunit Nav1.5/Scn5a was concentrated at the intercalated disks and along the cell membrane in PCs. Confocal images confirmed that Cntn2 is expressed in Nav1.5+ PCs; surface (Figure 4D) and subsurface (Figure 4E) optical slices showed that while coexpressed in the same cell, Cntn2 and Nav1.5 appeared to be targeted to distinct membrane domains with limited colocalization, most clearly seen in the surface view.

**Isolation and Functional Analysis of Cntn2-EGFP+ Myocytes**

To further analyze the properties of the Cntn2-expressing myocytes, we dissociated myocytes from the ventricles of adult Cntn2-EGFP+ transgenic mice. Cntn2-EGFP+ myocytes were readily seen under epifluorescence microscopy, with an elongated, rod-shaped phenotype typical of PCs, easily distinguishable from the nonfluorescent working ventricular myocytes (Figure 5A, left). Cntn2-EGFP+ cells did not express Cx43 (Figure 5A, center), the most abundant connexin of working myocytes, but were strongly positive for Cx40. This specificity of Cx40 expression was most obvious in a cluster of myocytes that included both EGFP/H11001 and EGFP/H1102 cells (Figure 5A, right).

Action potentials (APs) from Cntn2-EGFP+ myocytes were characterized by a distinct plateau in phase 2 (Figure 5B). APD50 and APD90 values were both significantly longer than those recorded from Cntn2-EGFP− myocytes (Table). Cntn2-EGFP+ myocytes also demonstrated, spontaneous electric oscillations in phase 2, which resulted in EADs (Figure 5C, top). In some instances (Figure 5C, bottom), Cntn2-EGFP+ myocytes also developed DADs at faster pacing rates (5 Hz); in contrast, none of Cntn2-EGFP− cells exhibited either EADs or DADs.

Confocal microscopy and fluorescence photometry with calcium-sensitive dyes were also performed to study intracel-
lular calcium dynamics. Compared with the Cntn2-EGFP−/H11002 working ventricular myocytes, Cntn2-EGFP+/H11001 cells displayed significantly slower kinetics of activation and relaxation (Figure 5D and 5E), with longer time to peak fluorescence, reduced peak rate of rise of fluorescence, and slower decay (Table). EGFP+/H11001 myocytes were also significantly more likely to develop spontaneous Ca2+/H11001 events (Figure 5F and 5G).

Discussion
We identified Contactin2 (Cntn2) as a novel marker that is preferentially expressed in the cardiac conduction system, including myocytes of the murine and canine PF networks. Contactins are a subgroup of cell adhesion molecules of the immunoglobulin superfamily. There are at least 6 contactins: Cntn1, Cntn2, BIG-1, BIG-2, NB-2, and NB-3; all include 6 immunoglobulin-like and 4 fibronectin III–like domains that are anchored to the plasma membrane by a glycosyl phosphatidylinositol moiety. Cntn2, also known as transiently expressed axonal glycoprotein 1 (TAG-1) in mouse and rat, transient axonal glycoprotein 1 (TAX1) in human, and axonin-1 in chicken, has been most fully characterized in the central and peripheral nervous sys-

Table. Functional Properties of Cntn2-EGFP− and Cntn2-EGFP+ Myocytes

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>RMP, mV</th>
<th>APA, mV</th>
<th>APD50, ms</th>
<th>APD90, ms</th>
<th>Time to Max, ms</th>
<th>Peak Rate of Rise, F/F0/s</th>
<th>Tau of Decay, ms</th>
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<tr>
<td>EGFP(−) (n=10)</td>
<td>−71.3±1.1</td>
<td>119.2±3.2</td>
<td>5.5±0.9</td>
<td>54.7±9.7</td>
<td>29.5±3.6</td>
<td>103.0±20</td>
<td>110.4±13.4</td>
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<tr>
<td>EGFP(+) (n=10)</td>
<td>−71.5±1.3</td>
<td>128.3±3.7</td>
<td>24.7±4.3</td>
<td>139.3±17.8</td>
<td>49.4±4.8</td>
<td>49.4±5.9</td>
<td>136.7±23.2</td>
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<tr>
<td>P value</td>
<td>0.87</td>
<td>0.08</td>
<td>0.00035</td>
<td>0.00057</td>
<td>&lt;0.000001</td>
<td>&lt;0.00001</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SEM. n=No. of cells. RMP indicates resting membrane potential; APA, AP amplitude; APD50, AP duration at 50% of repolarization, respectively. APDs are significantly longer for Cntn2-EGFP+ myocytes compared with Cntn2-EGFP− myocytes. Ca2+ transient measurements demonstrated that Cntn2-EGFP+ myocytes have significantly slower Ca2+-handling kinetics compared with Cntn2-EGFP− myocytes.
tens, where it is expressed in the membrane of neurons and myelinating glial cells.

We used several complementary strategies to confirm the transcriptional profiling data demonstrating expression of Cntn2 within the PF network. These included immunohistochemical studies of murine and canine heart, as well as analyses of 2 genetically engineered reporter mice, that is, Cntn2-lacZ knockin mice and Cntn2-EGFP BAC transgenic mice. Interestingly, although our initial transcriptional screen was focused on identifying transcripts enriched in PFs, our studies demonstrated expression of endogenous Cntn2 (and reporter gene expression) throughout the entirety of the adult CCS, including nodal as well as fast-conducting cells of the His-Purkinje network. This observation, coupled with our previous description of widespread expression of the CCS-lacZ reporter gene throughout both the proximal and distal CCS, suggests that despite the functional diversity that exists within the heterogeneous elements of the CCS, these disparate components of the conductive network share some commonality with respect to transcriptional regulation.

In this study, we took advantage of Cntn2-EGFP reporter mice because they provided us with a powerful approach to identify and functionally characterize individual Cntn2-expressing murine myocytes. These data, including patch-clamp recordings and measurements of intracellular calcium dynamics, were consistent with a PC phenotype. Importantly, our functional studies indicated a significantly greater propensity of single murine PCs to develop spontaneous intracellular calcium release events (Figure 5F) and afterdepolarizations (Figure 5G). These observations are consistent with the purported role of PCs in the genesis and maintenance of various arrhythmic syndromes, including inherited diseases such as catecholaminergic polymorphic ventricular tachycardia and idiopathic VF, as well as common acquired pathologies, such as post–myocardial infarction electrical instability.29,37 Importantly, the Cntn2-EGFP mice were able to characterize the ventricular conduction system at varying levels of resolution, including studies of single cells (as in the current study), as well as analyses of the complex branching structure of the Purkinje fiber network. These data should be of benefit in the formulation of increasingly sophisticated computational models of cardiac excitation.38,39

These studies do not yet address the specific function of Cntn2 in the cardiac conduction system, although there are tantalizing hints from studies in the developing and adult nervous system. During development, Cntn2 participates in neural cell adhesion and migration, neurite outgrowth and fasciculation, and axon path finding and myelination.40 Even more relevant for the interpretation of our findings in PCs is the role of Cntn2 in adult neurons, where it is involved in ion clustering with important implications for their electrophysiological properties.

Cntn2 is specifically involved in the clustering of delayed rectifier Shaker-type K+ channels, Kv1.1 and Kv1.2, and their Kvβ2 subunits at the juxtaparanodal region (JXP) of myelinated axons.27 Concentration of Kvs at the JXP and their segregation from domains enriched in voltage-gated Na+ channels (Navs), at the nodes of Ranvier plays an important role in limiting neuronal excitability by maintaining neuronal internodal resting potential or preventing rapid reexcitation.41 Molecular mechanisms of Cntn2-mediated Kv clustering in neurons involve trans interaction with other Cntn2 molecules on the glial membrane and heterophilic cis interactions with Caspr2, a member of the neurexin superfamily in the neuronal membrane (axolemma). Caspr2 expression in the heart has not yet been examined; however, in the nervous system, it influences the function of Cntn2 as demonstrated by studies showing that in Caspr2-deficient mice Cntn2 is unable to localize at the JXP and Kvs diffuse to the internodal region.27 Conversely, in Cntn2−/− mice, there is mislocalization of Caspr2 and Kvs at the internodal regions, demonstrating the interdependence of Cntn2 and Caspr2.42 These observations suggest that the function of Cntn2 in PCs could also be to regulate Kv clustering through its interaction with Caspr2, thereby modulating PC excitability. Our data showing that Cntn2 and Nav1.5 appear to be targeted to distinct membrane domains support this functional parallel in PCs and neurons. Additional colocalization studies using Cntn-EGFP mice will be required to explore potential molecular interactions between Cntn2, Kvs, Navs, and Caspr2 in PCs.

Previous studies have also shown that Cntn2 is able to induce cis activation and binding of L1-CAM to the adapter protein ankyrin, which, in neurons, is implicated in Nav clustering at the nodes (ankyrin-G).43 In cardiomyocytes, mutations in ankyrin-G binding domains of Nav1.5 that result in impaired Nav1.5 localization to the intercalated disks were recently implicated in the pathogenesis of ventricular arrhythmias.45–47 Furthermore, in the canine model of arrhythmia after infarction, ankyrin-G has been implicated in sodium channel remodeling of epicardial border zone cells.48 In light of its interaction with ankyrin, it is conceivable that Cntn2 may play a mechanistic role in ankyrin-dependent regulation of ion channel targeting and arrhythmogenesis.

In conclusion, in the present study we have identified Cntn2 as a novel marker that is robustly expressed throughout the specialized conductive system of the heart, including the distal PF network. This discovery has enabled us to visualize the CCS, to isolate individual PCs, and to characterize their distinct physiological properties. One can now envision numerous strategies to decipher the mechanistic role of the PF network in the genesis and maintenance of clinically significant cardiac rhythm disturbances. Moreover, by analogy to neuronal systems, we suggest that Cntn2 may regulate CCS network formation and the excitability of its component cells.

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Disclosures

None.

References


**CLINICAL PERSPECTIVE**

Purkinje fibers are the most distal component of the cardiac conduction system, and a growing body of literature suggests that the Purkinje fiber network plays a prominent role in the genesis of ventricular arrhythmias. Purkinje cell triggers have been implicated in ventricular tachycardia following myocardial infarction, in idiopathic ventricular fibrillation, and in inherited syndromes such as catecholaminergic polymorphic ventricular tachycardia and long-QT syndrome. In the present study we performed transcriptional profiling of isolated murine Purkinje fibers with the goal of discovering novel Purkinje-specific transcripts that might play a mechanistic role in regulating Purkinje cell electrophysiology and arrhythmogenesis. Through this strategy, we identified preferential expression of Contactin-2 (Cntn2) in Purkinje cells. Previously, Cntn2 has only been observed in the nervous system, where it is critical for regulating neuronal patterning, ion channel clustering, and neuronal excitability. These observations in neurons suggested a comparable role in regulating cellular electrophysiology in Purkinje cells. In fact, utilizing transgenic mice in which regulatory elements from the Cntn2 gene drive expression of a green fluorescent protein reporter gene, we were able to specifically isolate Purkinje cells from adult mice and demonstrate unique action potential properties as well as frequent afterdepolarizations. These observations are consistent with an important role of Purkinje cells as arrhythmic triggers. Moreover, with the tools now in hand to isolate highly enriched populations of Purkinje cells, one can envision novel approaches to identify antiarrhythmic targets that preferentially modulate Purkinje cell electrophysiology.
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SUPPLEMENTAL MATERIAL

Expanded Methods

**Microarray Profiling.** Purkinje fiber (PF) enriched samples were obtained by micro-dissecting the subendocardial layer of 6-8 month old *CCS-lacZ* murine hearts and PF depleted samples were micro-dissected from the sub-epicardial layer of the ventricular walls, using a dissection microscope (Zeiss) and 0.125 mm tungsten micro-needles. The tissue from three hearts was pooled together to generate one sample and three independent pooled samples of each tissue type were analyzed in transcriptional profiling performed on total RNA using the GeneChip 3’ IVT Express Labeling Kit (Affymetrix) to generate biotin labeled probes hybridized to Mouse Genome 430 2.0 Array chips, covering 39,000 mouse genes. Probe level was converted to expression values using both the Robust Multi-array Average (RMA) procedure. 415 probe sets for genes/mRNAs differentially regulated between PF-enriched versus PF-depleted samples were identified using triplicate sets of Robust multichip average (RMA)-normalized Affymetrix CEL files followed by feature selection algorithm Pavlidis Template Matching (PTM, \( p<0.05 \)) and thresholding at 33% for reproducible fold change in expression level between the two sample types.

**Immunohistochemistry** Mouse hearts or canine PFs were fixed in 4% PFA, then equilibrated in 10% sucrose and embedded in OCT. Cryosections, 6-10µM thick, were post-fixed for 10 min in 4% PFA (Cx40), permeabilized for 10 min in 0.5% Triton X-100 and blocked for 1 h at 37°C in 5% Normal Donkey Serum/0.1% Triton X-100/0.1% NP-40. Slides were then incubated overnight at 4°C with the primary antibody followed by
incubation for 1 hr at 37°C with the secondary antibody and mounted with Vectashield with or without the nuclear counter stain DAPI (VectorLabs). The permeabilization step was omitted for Cx40, HCN4 and Cntn2 single staining. Staining for β-galactosidase (βGal) and whole mount XGal staining were performed as previously described. For Cntn2/XGal serial staining, CCS-lacZ hearts were snap frozen in OCT, and sequential cryosections were stained for Cntn2 or fixed and stained for XGal.

Epifluorescent images were acquired with an Axiovert 200M inverted microscope (Zeiss) with 10x and 40x dry objectives, or a Semi SV 11 Apo dissection microscope, equipped for fluorescence (Zeiss). With both microscopes images were acquired with an AxioCam MRc camera (Zeiss) and AxioVision 4.6 software. Confocal images were acquired with a SP5 confocal laser system (Leica), using 20x (water) and 40x (dry) objectives, and Ar (488 nm line; green fluorescence) or Green HeNe (543 nM line; red fluorescence) lasers.

**Immunocytochemistry of canine PCs.** Freshly isolated PCs were seeded onto laminin–coated glass coverslips. After attachment the cells were fixed for 15 min with 4% PFA, permeabilized for 20 min with 0.7% Triton X-100, incubated for 30 min in 5% Normal Goat Serum blocking buffer. Cells were then incubated overnight at 4°C with the primary antibodies, followed by incubation for 1.5 h with the appropriate combination of secondary antibodies. Immunoreactivity was visualized by 488/568 excitation using a Zeiss LSM 510 scope with 100x objective.

**Antibodies.** The following primary antibodies were used: rabbit anti-HCN4 (Millipore), goat anti-Cntn2 (R&D Systems); mouse anti-sarcomeric actinin (Sigma); rabbit anti-Cx40 (Alpha Diagnostics); rabbit anti-βGal (5’-3’, Boulder, CO); rabbit anti-Nav1.5
rabbit (Alomone Laboratories, Israel). The following fluorophore-conjugated secondary antibodies were used: biotinilated goat anti-rabbit (Sigma); FITC avidin D (Sigma); Alexa Fluor 555 goat anti-rabbit (Invitrogen); FITC donkey anti-rabbit; TR-conjugated donkey anti-rabbit; TR or FITC donkey anti-goat; Cy3 donkey anti-mouse; FITC goat anti-mouse. Canine PCs were incubated with the following secondary antibody combination: FITC donkey-anti-Rabbit (Jackson ImmunoResearch) and Cy3-conjugated donkey anti-goat IgG. Antibodies were purchased from Jackson ImmunoResearch, unless otherwise stated, and were diluted in blocking buffer at a concentration of 2-10 µg/ml.

**Electrophysiological recordings in isolated ventricular myocytes**

Ventricular myocytes were isolated using an established enzymatic digestion protocol yielding to 60-80% rod-shaped, calcium-tolerant myocytes. Within 6 hours from isolation, laminin-coated dishes containing isolated ventricular myocytes were mounted on the stage of an inverted microscope. The myocytes were bathed with the solution containing (mmol/L): 140 NaCl, 4 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES and 5 glucose, pH adjusted to 7.4 with NaOH at 35°C. Transmembrane potentials were recorded in whole cell current mode as previously described. All signals were acquired at 5 kHz (Digidata 1322A, Axon Instruments) and analyzed with pCLAMP version 9.2 software (Axon Instruments). Patch electrodes had a resistance of 2–3 MΩ when filled with patch electrode solutions containing (mmol/L): 120 potassium aspartate, 20 KCl, 1 MgCl₂, 4 Na₂ATP, 0.1 GTP, 10 HEPES, 10 glucose, pH adjusted to 7.2 with NaOH. Myocytes
were electrically stimulated by intracellular current injection through patch electrodes using depolarizing pulses with duration of 3 ms and amplitude of 0.5-1 nA. Action potential duration (APD) was measured at 90% and 50% of repolarization (APD$_{90}$ and APD$_{50}$). DADs were defined as phase 4 positive (depolarizing) deflections of the membrane potential. EADs were defined as positive (depolarizing) oscillations occurring during phase 2 or 3 of action potential.

**Ca$^{2+}$ imaging.** Isolated myocytes from *Cntn2-EGFP* transgenic mice were loaded with the membrane-permeant Ca$^{2+}$ indicator dye Rhod-2/AM. Cells were placed in solution with 5 µM Rhod-2/AM (Invitrogen) at room temperature for 20 min. The loading solution was removed, and cells was washed and equilibrated in fresh Tyrode’s solution for 30 min to allow deesterification of the dye before recording. Epifluorescence light was used to identify the $EGFP^+$ and $EGFP^-$ cells. Ca$^{2+}$ images were acquired using a 63x water immersion objective (N.A.=1.2) with a Leica SP5 confocal microscope in line-scan mode (400Hz). Rhod-2 was excited at 540-nm. Emission was collected at >560-nm. During the experiments, cells were field stimulated with 1 Hz at least 1 min to reach a steady-state of SR Ca$^{2+}$ loading. In some experiments, the Ca$^{2+}$ transients were measured by microfluorimetry. Freshly isolated myocytes were incubated with 2µM Indo-1/AM (Invitrogen) at room temperature for 15min. Then the loading solution was replaced with fresh Tyrode’s solution for 30 min to allow deesterification of the dye before recording. The Indo-1 loaded cells were illuminated by a Xenon lamp (excitation was 340nm) and the dual emission light (405-nm and 495-nm) was collected by two separate PMT tubes. The fluorescence ratio was calculated by Ionwizard (IonOptix, Milton, MA).
References


Table S1. Primers specific for mouse genes

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<sup>a</sup> conventional RT-PCR; <sup>b</sup> QRT-PCR
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