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The junctophilin family of proteins: From bench to bedside

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Abstract

Excitable tissues rely on junctional membrane complexes to couple cell surface signals to intracellular channels. The junctophilins have emerged as a family of proteins critical in coordinating the maturation and maintenance of this cellular ultrastructure. Within skeletal and cardiac muscle, junctophilin-1 and junctophilin-2, respectively, couple sarcolemmal and intracellular calcium channels. In neuronal tissue, junctophilin 3 and junctophilin 4 may have an emerging role in coupling membrane neurotransmitter receptors and intracellular calcium channels. These important physiologic roles are highlighted by the pathophysiology which results when these proteins are perturbed and a growing body of literature has associated junctophilins with the pathogenesis of human disease.

Keywords

calcium; excitation contraction coupling; JPH; junctophilin; mutation

AN EMERGING ROLE IN THE PATHOPHYSIOLOGY OF EXCITABLE CELLS

Since the sentinel discovery of the junctophilin (JPH) family of proteins a decade ago[1], a rapidly progressing body of literature has linked these proteins to critical roles in all excitable cells with implications for human physiology and pathophysiology. Through maintaining critical subcellular architecture, and an emerging function as a direct regulator of calcium-handling proteins, impaired JPHs have been implicated in a number of human

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CONFLICTS OF INTEREST

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diseases. Investigations into the role of JPHs in cellular physiology have elucidated novel mechanisms of skeletal muscle myopathy, cardiomyopathy, heart failure, arrhythmia, behavioral and learning deficits, motor control, and Huntington Disease-like pathology. Future investigations hold the promise of novel therapeutic targets and molecular interventions for a number of these diseases.

PROTEIN PHYLOGENY AND STRUCTURE

Junctophilins are members of a family of junctional membrane complex (JMC)-associated proteins found in all excitable cells from striated muscle to neurons. JPHs were originally discovered using an immuno-proteomic monoclonal antibody library. This library was created from mice immunized with rabbit skeletal muscle membranes taken from transverse tubules (T-tubules) and muscle triads[2]. JPHs contain specific protein domains which define them as a family, including eight amino-terminal membrane occupation and recognition nexus (MORN) motifs separated by a joining region, followed by an alpha helix, divergent, and a C-terminal transmembrane motif that anchors the protein into the endoplasmic reticulum (ER) or sarcoplasmic reticulum (SR)[1, 3]. The protein topology for each JPH isoform is depicted in Figure 1.

MORN motifs have been found to be highly conserved across all JPH isoforms, as well as across various species, and demonstrate a consensus sequence of YxGxWxxGxRHGYG[4]. Initially identified by BLAST analysis, the eight MORN motifs in JPH are separated by a ‘joining region’ which links MORN motifs I–VI to VII and VIII. As a whole, the MORN motifs are thought to traffic and bind JPHs to the plasma membrane. This hypothesis was based on early experiments which truncated the carboxy-terminus of the protein and resulted in localization of the MORN motif-containing amino terminus strictly to the plasma membrane[1]. Further, homologous MORN motifs in plants, such as *A. thaliana*, have high affinity for membrane phospholipids such as phosphatidic acid and somewhat lower affinity for membrane phosphatidylinositol (PI)4P and PI(4,5)P2[5]. In addition to JPHs, multiple MORN motifs are found in several other classes of proteins including histone-lysine N-methyltransferase and phosphatidylinositol-4-phosphate 5-kinase proteins[1, 6].

The carboxy-terminal membrane-spanning domain of JPH is believed to embed the protein into intracellular membranes such as the ER or SR[1, 3]. This domain is highly conserved across species and isoforms, and *in silico* secondary structure analysis as well as hydrophobicity analysis predict the presence of this domain[1, 4]. Linking the MORN motifs and the membrane-spanning domain are two domains, the alpha-helical domain and the divergent region. Highly conserved across JPH isoforms, *in silico* analysis predict multiple alpha-helical stretches within the primary sequence of this domain. Spanning approximately one hundred amino acids, it is thought to bridge the gap between the plasmalemma/sarcolemma and the ER/SR[1, 3, 7]. Given the possible alpha-helical structure, this domain may provide mechanical elasticity to regulate dyad distance[1, 4]. Conversely, the divergent region derives its name from the relatively low primary sequence conservation between the various JPH isoforms, and its role is unclear at present[4]. Overall, these presumed functional domains allow for JPHs to play critical roles in the maintenance

of cellular ultrastructure, particularly, the interface between intracellular membranes such as the ER/SR and the plasmalemma/sarcolemma.

Initial studies in rodent models as well as in-depth bioinformatic and phylogenic analysis have identified four major JPH family members in higher chordate organisms, including humans[4]. Each is expressed preferentially in various excitable tissues and include skeletal muscle *JPH1*-encoded junctophilin type 1 (JPH1), cardiac muscle *JPH2*-encoded JPH2, and neuronal tissue *JHP3*-encoded JPH3, and *JPH4*-encoded JPH4. A phylogenic tree generated from multiple JPH isoforms across a diverse group of species is depicted in Figure 2.

CALCIUM SIGNALING IN STRIATED MUSCLE

A major role of Ca^{2+} within muscle is the initiation and coordination of myofilament contraction[8–11]. In cardiac myocytes, Ca^{2+}-mediated contraction signaling begins with the opening of voltage-gated L-type Ca^{2+} channels (LTCC) at the sarcolemma in response to membrane excitation. LTCC Ca^{2+} influx down its electrochemical gradient triggers a larger Ca^{2+} release from the SR via the intracellular Ca^{2+} release channel ryanodine receptor type 2 (RyR2) in a process known as Ca^{2+}-induced Ca^{2+}-release (CICR, see Glossary). In this manner, Ca^{2+} traveling across the cardiac dyad triggers the opening of RyR2 and release of SR Ca^{2+} without direct coupling between the LTCC and RyR2. This process serves as the molecular initiator for ATP-expending myofilament-based mechanical contraction of the heart. Contraction is terminated by active removal of Ca^{2+} from the cytosol into the SR via the action of the sarcoplasmic reticulum calcium ATPase (SERCA2a) or removal from the cell by the sodium-calcium exchanger (NCX1). While skeletal muscle is analogous in many ways, store Ca^{2+} release is directly triggered by alterations in sarcolemmal potential, a process known as voltage-dependent calcium-release[12]. During this process, voltage changes directly trigger release of store Ca^{2+} through direct coupling of the LTCC and RyR1[13, 14].

JUNCTOPHILIN TYPE 1 AND SKELETAL MUSCLE

JPH1-encoded JPH1 is the major JPH family member expressed in skeletal muscle but also demonstrates a low level of expression in the heart. *JPH1* contains 5 coding exons localizing to 8q21 and encodes a 658 amino acid 90 kDa protein in humans[15]. *JPH2*-encoded JPH2 is also expressed in skeletal muscle and is the predominant cardiac isoform[16]. JPH1 maintains the critical ultrastructural geometry of the skeletal muscle triad which is comprised of three independent membranous structures including the T-tubular invagination of the sarcolemma flanked on two sides by cytosolic SR[17]. Loss of JPH1 expression has been shown to reduce the number of intact triads and to deform intact triads[18]. A detailed explanation of skeletal muscle triads, cardiac dyads, and the junctional membrane complex can be found in Box 1.

Box 1

Subcellular Architecture and the Junctional Membrane Complex

Precise subcellular architecture of intracellular membrane and associated proteins is required for effective transmission of membrane excitation to mechanical contraction of

skeletal and cardiac muscle. To facilitate this, the sarcolemma of striated muscle cells has deep, finger-like invaginations that penetrate the interior of the myocyte. Running parallel along these T-tubules are portions of the SR which are so-called junctional SR. In skeletal muscle, the SR runs alongside a shared portion of the T-tubule on two opposing sides which create a triad of membranes observed on microscopy. In cardiac muscle, a single junctional SR runs alongside the T-tubule over a given portion of the T-tubule appearing as a dyad. The distance of the triad or dyad is carefully controlled and is critical for efficient movement and signaling of ions, particularly Ca^{2+}. In the cardiac cell, it is movement of extracellular Ca^{2+} across the sarcolemma and the cardiac dyad which triggers CICR and allows for efficient EC coupling. A host of proteins, including JPHs, are crucial to the development and maintenance of this architecture. Further, the proteins which localize to the interface of these membranes and are responsible for Ca^{2+} cross talk are referred to as the JMC.

In addition to a structural role, JPH1 directly binds and regulates a number of proteins within the skeletal muscle JMC. JPH1 directly interacts with the sarcolemmal LTCC through a binding domain on JPH1 spanning from amino acids 232–369 within the joining region domain of the protein. In addition, JPH1 directly interacts with sarcolemmal *CAV3*-encoded caveolin type 3 (Cav3)[19]. JPH1 also directly interacts with the skeletal muscle specific, *RYR1*-encoded ryanodine receptor type 1 (RyR1) which mediates release of Ca^{2+} stored within the SR. This interaction is mediated through highly reactive thiol groups which are sensitive to oxidative insult, and changes in this interaction can alter gating of RyR1. Overall, these interactions suggest a mechanism through which release of SR-stored Ca^{2+} via RyR1 is mediated by a direct interaction with JPH1 in an oxidation-dependent fashion[20]. A schematic depicting JPH1 within the skeletal muscle triad is depicted in Figure 3.

In vitro, colocalization of LTCC and RyR1 has been demonstrated by immunofluorescence into discrete areas along C2C12 murine skeletal myoblasts. This localization is disrupted following expression silencing of JPH1 and is associated with impaired LTCC Ca^{2+} influx[19]. Further, JPH1 expression silencing *in vitro* decreases store-operated Ca^{2+} entry (SOCE) which balances net Ca^{2+} loss from intracellular Ca^{2+} stores[18]. This results in reduced SR-stored Ca^{2+} release via RyR1[18]. Follow-up studies suggest that impaired SOCE may occur through disruption of stromal interaction molecule 1 (STIM1) and calcium release-activated calcium modulator 1 (ORAI1)[21]. In this model, depletion of SR-store Ca^{2+} allows for STIM1 oligimerization within the SR and interacts with ORAI1, which allows for SOCE through the sarcolemma. This interaction is disrupted with loss of JPH1 expression impairing the ability of the myocyte to increase sarcolemmal Ca^{2+} influx to balance SR Ca^{2+} depletion. RyR1 function is also regulated by the transient receptor potential channel canonical type 3 (TRPC3)[22]. Knockout of TRPC3 in primary skeletal myotubes demonstrate impaired RyR1 EC coupling gain and SR Ca^{2+} release with intact SR store Ca^{2+} levels. Taken together, these results support the role of JPH1 in regulating the skeletal muscle triad ultrastructure and the JMC. This role is critical to maintain efficient EC coupling gain, SR store Ca^{2+} release, and SOCE.

While the precise mechanism of SOCE remains controversial, a body of evidence suggests that SOCE plays a minimal role, if any, during physiologic muscle contraction. As the majority of cytosolic Ca^{2+} is removed from the cytosol by SERCA into the SR, a relatively small amount of Ca^{2+} is extruded from the myocyte[23]. Conversely, during non-physiologic experimental conditions, or under states of muscle fatigue, SOCE plays a critical role in maintaining store Ca^{2+} homeostasis[24, 25]. Further, since there is minimal net extravasation of cytosolic Ca^{2+} from intact skeletal myocytes during relaxation, SOCE is seen more prominently with *in vitro* myoblasts, such as C2C12 cells [26]. Thus, additional studies are needed to further elucidate the role of JPH1 in regulating SOCE utilizing *in vivo* models of intact, adult skeletal myocytes.

Skeletal Muscle Myopathy and Damage

Mice lacking JPH1 die shortly after birth due to defective suckling. This early mortality cannot be rescued by placement of an orogastric tube for feeding, suggesting that milk aspiration or respiratory failure, likely due to pharyngeal muscle and diaphragmatic dysfunction, contributes to pup death[27]. Skeletal myocytes isolated from these mice demonstrate fewer apparent JMCs, reduced contractile force, and reduced Ca^{2+}-mediated twitch tension[27]. In addition, this perinatal mortality is associated with failure of normal triad development as well as disruption and vacuolization of normal SR structure[28]. These studies suggest that JPH1 plays a key role in skeletal muscle EC coupling and that disruption or impairment of JPH1 function is associated with poor skeletal muscle function through a loss of cellular ultrastructure and triad development.

JPH1 expression has been shown to decrease after skeletal muscle damage from eccentric contraction, a type of contraction in which the muscle elongates under tension due to an opposing force which is greater than the traditional contractile force generated by the muscle[29]. This loss of expression coincides with loss of EC coupling, and gradual return of JPH1 protein levels correlates with recovery of EC coupling and force generation. Further investigation found that increased skeletal muscle contraction, as well as directly increasing cytosolic Ca^{2+} levels to supra-physiologic levels, result in calpain-mediated proteolysis of JPH1[30]. Specifically, JPH1 is cleaved proximal to the C-terminus creating a 75 kDa and a 17 kDa fragment with a loss of expression of full-length protein. Interestingly, aberrant JPH1 proteolysis was identified in a murine mouse model of muscular dystrophy providing an association with the development of primary muscle disease[30]. These findings suggest that eccentric contraction, prolonged muscle contraction, and other mechanical stress may develop impaired contractile force and EC coupling secondary to loss of JPH1 function.

JUNCTOPHILIN TYPE 2 AND CARDIAC MUSCLE

The *JPH2* gene contains five coding exons localizing to 20q13.12 and encodes a 696 amino acid 74kDa protein JPH2 in humans[15]. JPH2 is the major JPH family member expressed in cardiac muscle and shares significant primary sequence identity and functional parallels with JPH1 in skeletal muscle. In contrast to skeletal muscle, which demonstrates triadic ultrastructure, cardiac muscle characteristically has dyadic ultrastructure whereby a span of junctional SR runs parallel to one sarcolemmal t-tubular membrane at a given location[31,

32]. JPH2 plays a critical role in maintaining this dyadic structure and associated effective Ca^{2+} signaling. For a schematic depicting JPH2 within the cardiac dyad see Figure 4.

JPH2 null mice, while embryonic lethal, demonstrate a 90% increase in the distance of the cardiac dyad as well as vacuolization of the SR[1]. This disruption in cellular ultrastructure was associated with random cardiomyocyte contraction and irregular Ca^{2+} transients which occurred spontaneously without Ca^{2+} influx through the LTCC. Further, Ca^{2+} transients in JPH2 null mice are significantly reduced in amplitude perhaps due to the disruption of the SR ultrastructure[1]. From these early studies, JPH2 was originally viewed as a structural protein serving as a molecular tether which maintained the cardiac dyad ultrastructure required for effective CICR and Ca^{2+} signaling. Recent studies have added complexity to the function of this protein.

Development of the Junctional Membrane Complex and the T-Tubule

JPH2 plays a key role in cardiomyocyte development and differentiation. While it is possible that the amino-terminal MORN motifs of JPH2 might directly interact with the inner leaflet of the sarcolemma, JPH2 has also been shown to bind Cav3, a protein which localizes to the sarcolemma[33]. Cav3 is necessary for the formation of “little cave”-like invaginations of the cardiomyocyte sarcolemma called caveolae as well as regulation of several cardiac ion channels including the LTCC, and various K^+ channels[34, 35]. Immunofluorescent localization of the LTCC, RyR2, Cav3, and JPH2 in rat myocytes demonstrates a temporal correlation between development of influx of Ca^{2+} from the $LTCC_a$, CICR, and T-tubule formation[36]. While expression of Cav3 and RyR2 was observed early in development, JPH2 expression was not seen until day 15 and was associated with formation of the T-tubule and maturation of CICR. Further, embryonic stem cell-derived cardiomyocytes with reduced JPH2 expression demonstrate perturbed cardiac development and decreased Ca^{2+} transients that are sporadic and irregular. These cells also demonstrated disordered myofilament production, perturbed energy-generating capacity, and Ca^{2+} dysregulation, which culminated in fewer spontaneously contractile myocytes[37].

These studies in developing cardiomyocytes correlate well with studies in differentiated cardiac cells. JPH2 expression silencing in cardiac cells *in vitro* has been shown to impair CICR with decreased Ca^{2+} transient amplitude and blunting of spontaneous generation of Ca^{2+} transients[38]. These findings were seen with unchanged expression of the LTCC, RyR2, NCX, SERCA, and phospholamban and suggest altered function of RyR2 rather than altered protein expression. Induced JPH2 knock-down in adult mice demonstrated RyR2 hyperactivity with increased spark frequency despite impaired CICR and EC coupling gain[7]. Co-immunoprecipitation showed RyR2 directly binds JPH2 suggesting a role in regulation of RyR2 gating. Further, single molecule super-resolution microscopy in rat ventricular myocytes identified RyR2 clusters in close proximity to similarly shaped clusters of JPH2 with approximately ~80% colocalization[39]. These studies suggest that direct binding of JPH2 to RyR2 is necessary for proper RyR2 gating and loss of JPH2 expression results in reduced EC coupling gain during CICR and increased RyR2 Ca^{2+} leakage during diastole.

In addition to Ca^{2+} signaling, JPH2 plays a key role in the development and stability of myocyte ultrastructure. Mice with decreased levels of cardiac JPH2 showed defective postnatal T-tubule maturation, while mice over-expressing JPH2 had accelerated T-tubule maturation by postnatal day 8[40]. Further, independent studies utilizing acute RNAi-mediated knockdown of JPH2 in cultured cardiomyocytes have shown T-tubule disorganization[41]. Taken together, these studies suggest that JPH2 plays both a structural as well as protein-regulatory role within the cardiomyocyte. Reflecting this critical role, perturbations in JPH2 expression or mutations result in cardiac pathology.

Cardiomyopathy and Arrhythmogenesis

Primary myocardial diseases, such as cardiomyopathies, are a common cause of morbidity and mortality worldwide[42]. JPH2 has been linked to the development of various types of myocardial disease in both rodent models as well as humans. Murine genetic models of dilated cardiomyopathy (DCM) and hypertrophic cardiomyopathy (HCM), demonstrate reduced protein expression[33]. In addition, a rat model of aortic-banding pressure-induced hypertrophy similarly demonstrated reduced JPH2 expression and was associated with disruption of the cardiac dyad and junctional SR surface[43]. *In silico* modeling of the effect of this disrupted junctional interaction between LTCC and RyR on CICR predicts slow Ca^{2+} transient generation and reduced transient amplitude[44]. These studies suggest that loss of JPH2 expression, and the resulting disruption in Ca^{2+} transient generation, are associated with early molecular remodeling that may precede pathologic cellular remodeling.

Based on these findings, a series of studies were conducted to determine whether loss of JPH2 expression could drive cardiomyopathic disease. Knock-down of JPH2 in immortalized cardiac cells induced cellular hypertrophy, up-regulation of several hypertrophy markers including skeletal actin, atrial natriuretic factor, brain natriuretic factor, myosin heavy chain, regulator of calcineurin 1-exon 4 splice isoform, and was associated with reduced transient amplitude[38]. *In vivo*, conditional cardiac knockdown of JPH2 in mice rapidly leads to heart failure and early mortality[7]. These mice demonstrated increased cardiac mass with dilated ventricles and reduced fractional shortening as well as reduced CICR due to reduced EC coupling gain[7]. These murine model findings were supported by complementary human clinical studies where myocardium taken from surgical resection of HCM patients with left ventricular outflow tract obstruction demonstrated reduced, and in some cases undetectable, levels of JPH2[38]. In addition, cardiomyocytes isolated from patients with DCM and ischemic cardiomyopathy were observed to have reduced and mislocalized T-tubule-SR junctions with reduced JPH2 expression[44].

Based on the observation that altered JPH2 expression is associated with cardiomyopathic disease and heart failure, several studies have sought to identify whether mutations in the gene encoding JPH2 might be similarly associated with disease. The sentinel study in this regard identified three mutations localizing to the amino terminus first MORN motif domain and the linker domain of JPH2[45]. These mutations include JPH2–S101R, Y141H, and S165F. Each mutation was identified in a Caucasian individual who demonstrated clear clinical disease yet did not have a mutation in a gene traditionally associated with the development of HCM[45–47]. As with reduced JPH2 expression, these mutations were

found to reduce CICR amplitude and disrupt cellular ultrastructure resulting in cellular hypertrophy when over-expressed[45]. Two additional genetic variants localizing to the divergent domain, JPH2-R436C and G505S, were reported in a small cohort of Japanese patients with HCM[48]. Further genetic analyses have identified each of these mutations in control cohorts of ostensibly healthy individuals without HCM, thus it is likely these variants are polymorphisms without clinical significance[49]. Recently, a JPH2 mutation has been identified in a small family with HCM and prominent clinical arrhythmia[50]. The JPH2-E169K mutation was associated with development of supraventricular arrhythmias in a small multi-generational family. A murine model of this mutation exhibited a higher incidence of pacing-induced atrial fibrillation secondary to abnormal spontaneous Ca^{2+} waves and increased spark frequency resulting from decreased RyR2-JPH2 interaction[50]. These genetic studies suggest that JPH2 may function beyond JMC structural integrity and may also have a regulatory role on RyR2 and perhaps other JMC-associated proteins. Finally, while the frequency of JPH2 mutations among patients with HCM is rare (<1%), loss of JPH2 expression may demonstrate a common feature of hypertrophic and arrhythmic remodeling[49].

Skeletal muscle myopathy linked to JPH2 mutations

While JPH2 mutations have been associated with cardiomyopathy and arrhythmias, a small number of studies have linked these mutations to perturbed skeletal muscle function *in vitro*. Over-expression of the JPH2-S165F mutation in primary murine skeletal myotubes resulted in hypertrophy, reduced CICR, and reduced SR Ca^{2+} release via RyR1[51]. Immunoprecipitation assays suggest that the S165F mutation impairs protein kinase C-mediated phosphorylation resulting in impaired binding to TRPC3[51]. TRPC3 had been previously found to modulate the function of RyR1 through a direct interaction with JPH2 in skeletal muscle and binds JPH2 near the joining region[52, 53]. Thus, the S165F mutation is believed to lead to skeletal myocyte hypertrophy through impaired regulation of RyR1 function. JPH2-Y141H has also been linked to skeletal muscle hypertrophy and altered Ca^{2+} signaling[54]. Over-expression of JPH2-Y141H in primary skeletal myotubes demonstrated increase in cellular diameter and reduced EC coupling; however, unlike S165F, impairment of RyR1-mediated SR Ca^{2+} release was not observed. Rather, increased SOCE via Orai1 and increased resting Ca^{2+} levels was observed. These findings suggest that the JPH2-Y141H mutation is associated with skeletal muscle hypertrophy through a mechanism independent of RyR1 impairment. Interestingly, the initial probands hosting the JPH2-S165F and JPH2-Y141H mutations demonstrated no clinical evidence of skeletal muscle myopathy such as muscular dystrophy. Further, these studied have not been replicated using *in vivo* models and the clinical relevance of these mutations on skeletal muscle remains unclear at present.

JUNCTOPHILIN TYPE 3 AND 4 AND NEURONAL TISSUE

JPH3 (also referred to as JP3 elsewhere in the literature) and JPH4 are primarily expressed within the neurons of the brain. While the precise role of these proteins remains enigmatic, an emerging body of evidence suggests that neuronal JPHs may play roles in mediating balance and motor control through maintenance of efficient Ca^{2+} signaling. JPH3 knock-out

mice demonstrate reduced balance and impaired motor coordination at 3 months of age without overt alterations in brain morphology or significant defects in molecular signaling[55]. While JPH1 and JPH2 knock-out mice demonstrated disrupted ultrastructure of the skeletal myocyte triad and cardiomyocyte dyad, respectively, JPH3 knockout mice had no discernible disruption in neuronal cellular architecture. In addition, these mice demonstrated no apparent difference in purkinje cell action potentials and normal synaptic function[55]. A follow-up study utilizing JPH3 knock-out and hemizygous knock-out mice aged to 6 and 9 months of age, identified progressive defects in neuromuscular strength, coordination, and balance which was greater in the knock-out mouse and progressive over time[56]. JPH4 localizes to spatially discrete areas in the brain compared to JPH3; however, the knockout mouse has no discernible phenotype[57, 58]. Detailed intracellular Ca^{2+} signaling studies in these mice have not been done to date, nor has a comprehensive analysis of all major functional regions of the brain been done to determine whether a more robust phenotype may be identifiable. Despite these shortcomings, these results suggest that JPH3 and 4 are not required for cellular architecture stabilization and brain development individually which raises the possibility that the function of these isoforms may be redundant.

Supporting this hypothesis, JPH3/4 double knock-out mice demonstrate disrupted intracellular Ca^{2+} signaling[58]. Indeed, disrupted communication between plasmalemmal Ca^{2+} entry via the *N*-methyl *D*-aspartate (NMDA) glutamate receptors and intracellular neuronal RyRs as well as the small-conductance Ca^{2+}-activated potassium (SK) channels was noted in hippocampal neurons[58]. Under normal physiologic conditions, these plasmalemmal and intracellular channels are necessary for intracellular Ca^{2+} signaling and proper neuronal function[58]. In addition to this molecular defect in hippocampal neurons, JPH3/4 double knock-out mice also demonstrate a similar impairment in Ca^{2+} signaling of purkinje cells[59]. Specifically, purkinje cells obtained from double knock-out mice have impaired Ca^{2+} crosstalk between plasmalemmal P/Q-type voltage gated channels and intracellular RyR channels impairing SK channel-mediated after-hyperpolarization[59]. These mice demonstrate reduced memory and exploratory activity, as well as irregular hind limb reflexes ultimately culminating in death of young mice within five weeks of birth[58]. While the full mechanistic implications of this defective Ca^{2+} signaling has yet to be clarified, these early studies raise the possibility that defective store Ca^{2+} release by RyR isoforms may be associated with neuronal JPH loss. While little is known about the role of RyR within neuronal tissue, RyR3 has canonically been viewed as the major isoform of the brain while RyR1 and RyR2 are also expressed[60, 61]. While the specific RyR isoform responsible for ER Ca^{2+} release has not been clarified for the numerous neuronal cell subtypes in the brain, it is hypothesized that RyR isoforms may by neuronal subtype-specific[62, 63]. In addition, recent evidence has suggested that neuronal LTCCs may couple with RyR1 in a manner analogous to voltage triggered-RyR1 opening in skeletal muscle. Indeed, heterozygous knock-in mice harboring a known RyR1-I4895T mutation were noted to have significantly impaired voltage-induced Ca^{2+} release of store Ca^{2+} in addition to skeletal muscle myopathy[64]. These recent studies raise the possibility that molecular uncoupling of voltage-sensitive Ca^{2+} channels from neuronal RyRs in JPH3/4

deficient mice may mirror to the uncoupling of SOCE from RyR1 in skeletal muscle with loss of JPH1. JPH3 and 4 within neurons are depicted in Figure 5.

Huntington Disease-Like Syndrome

JPH3 was the first member of the junctophilin family to be implicated in the pathogenesis of human disease. An insidiously progressive neurodegenerative disease, Huntington's disease (HD) is associated with development of involuntary and uncoordinated muscle movements (chorea) and subcortical dementia[65]. The etiology of this disease is traditionally believed to be a CAG trinucleotide repeat expansion within the gene *HTT* which encodes the huntingtin protein[66]. This trinucleotide repeat results in the insertion of a polyglutamine tract within the huntingtin protein. While this mutation represents the classic cause of HD, a subset of patients are negative for mutations in this gene and yet are clinically indistinguishable from patients with mutations in *HTT*. These mutation-negative individuals are considered Huntington's disease-like syndrome phenocopies. Some patients with HD-like syndrome were found to host octapeptide repeat expansion in the *PRNP*-encoded prion protein and have been designated HDL1[67]. In 2001, a trinucleotide CAG/CTG repeat expansion was identified in a family with HD-like syndrome that was negative for mutations in *HTT* that localized to an alternatively-spliced exon of *JPH3*[68, 69]. This has led to the labeling of *JPH3* as the second HD-like associated gene (HDL2). While large studies estimating the frequency of *JPH3* mutations in HD or HD-like syndromes have not been done, a study examining nine independent series of patients with HD-like syndrome estimated that 0–15% of patients who are negative for mutations in *HTT* will host mutations in *JPH3*[70]. A recent study of a cohort of Venezuelan subjects with HD-like syndrome found that approximately 25% (4/16) hosted *JPH3* expansion mutations[71]. These numbers represent early estimates derived from small studies; however, it is generally accepted that *JPH3* mutations are a relatively rare cause of HD-like syndrome.

While mutations in *JPH3* underlie a relatively small proportion of individuals with HD-like syndrome, multiple independent cohorts have identified this disease association particularly in individuals of African ancestry[70, 72–74]. Individuals with HDL2 demonstrate a triad of symptoms marked by abnormal movements (most often chorea), dementia, and psychiatric disturbances, and the number of trinucleotide repeats is inversely proportional to the age of symptom onset[70]. Recent familial studies have added additional complexity to the clinical phenotype associated with *JPH3* mutations. Several large, multi-generational families hosting *JPH3* trinucleotide repeat expansions demonstrated severe akinetic-rigid syndrome with a relatively minor degree of chorea[71, 75]. Despite the possible emergence of distinct clinical presentations of individuals with HDL2, there has been no reproducible genotype-phenotype correlation which might explain why a given family demonstrates hyperkinetic chorea symptoms while another develops akinetic rigid syndrome symptoms.

While the precise mechanism of disease pathogenesis in HDL2 remains unknown, some progress has been in made in clarifying the pathogenic mechanism of the JPH3 trinucleotide repeat expansion. Fluorescent *in situ* hybridization (FISH) analysis of neuronal specimens obtained from HDL2 patients at autopsy demonstrated RNA foci within the neurons which were absent in traditional HD patients[76]. FISH for CAG/CTG repeat expansion of the

mutated JPH3 colocalize to these areas of RNA foci. Further, over-expression of the alternatively spliced exon 2A containing the repeat expansion resulted in cellular toxicity *in vitro*[76]. These studies suggest that the trinucleotide repeat expansion of JPH3 may result in aberrant RNA transcription and formation of toxic RNA foci. This possibility is supported by murine models over-expressing the trinucleotide repeat that demonstrate accelerated development of motor deficits as well as neurodegeneration[77]. Recent *in vitro* studies have raised the possibility that additional anti-sense transcription in an open reading frame within the *JPH3* gene locus may also play a role in neuronal toxicity[56]. While this possible anti-sense transcript has yet to be identified *in vivo*, RNA toxicity remains an interesting possible mechanism of neuronal dysfunction. Taken together, these studies demonstrate that neuronal toxicity and death may be occurring through multiple mechanisms including loss of JPH3 expression or generation of toxic RNA.

CONCLUDING REMARKS

The JPH family of proteins is critical for proper Ca^{2+} signaling within excitable cells, and pertubation results in cellular dysfunction that can manifest in clinical disease. While initially thought to be structural proteins holding together ultrastructural cellular architecture, it has become clear that striated muscle JPHs also modulate the function of Ca^{2+}-handling proteins and are needed for physiologic Ca^{2+} signaling. Perturbations in JPH expression, or function-impairing mutations, can alter intracellular Ca^{2+} signaling resulting in myopathic disease secondary to pathologic cellular remodeling as well as arrhythmogenesis. An emerging body of literature raises the possibility of a similar role in neuronal tissue such as the brain, and mutations in neuronal-specific JPHs have been linked to neurodegenerative disease. While the number of patients with clearly defined mutations in JPH genes remains small, understanding this class of proteins may provide significant understanding to the way in which excitable cells function and how to intervene when they fail. A list of outstanding questions and possible future directions for investigation is located in Box 2.

Box 2

Outstanding Questions

- Do JPH isoforms undergo post-translational modifications such as phosphorylation, and if so, what is the effect of these modifications on Ca^{2+} signaling within the cell?
- What is the role of *JPH1* mutations, if any, in the pathogenesis of skeletal muscle myopathies?
- Are there additional binding partners of striated muscle-specific JPH1 and JPH2 which may play roles in the development of myopathic disease?
- Are there additional binding partners of cardiac JPH2 which may play roles in arrhythmic disease?
- How do mutations in various functional domains of JPH1 and JPH2 create differing disease phenotypes?

- What are physiologic and pathophysiologic triggers for proteolytic degradation of JPH1 and 2, and can this form of post-translational modification be extended to JPH3 and 4?
- Can over-expression of JPH2 in cardiac cells, or JPH3/4 in neuronal cells, be protective against cardiomyopathic and neurodegenerative processes, respectively?
- What is the mechanism of disease pathogenesis triggered by the JPH3 trinucleotide expansion in patients with Huntington's Disease-Like syndrome?
- What is the role of mutations in JPH4, if any, in the pathogenesis of neurodegenerative disease?
- Are there other brain or neuronal diseases that can be linked to perturbed JPH expression or genetic mutations?
- Do JPH3 and/or 4 play a role in learning and memory?

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GLOSSARY

Akinetic-rigid syndromes	A constellation of symptoms characterized by a paucity of voluntary movements which, when present, are slow, and associated with increased muscle tone/rigidity
Alternative splicing	A molecular process during which the messenger RNA transcribed from a single gene can be processed differently to create various mature mRNA sequences which are then ultimately translated into different proteins
Calcium-induced calcium-release (CICR)	Release of a relatively large amount of stored intracellular calcium is triggered by a relatively small amount of calcium, such as from extracellular calcium moving intracellularly
Chorea	A neurologic disorder characterized by abnormal involuntary movements which are generally abrupt, irregular, and non-stereotyped in nature. It is derived from the Greek word meaning "dance"
Diastole	Relaxation of the heart or cardiac myocyte
Dilated cardiomyopathy (DCM)	Enlargement and dilation of the heart associated with impaired function and weak contraction

Excitation-contraction coupling (EC coupling)	Mechanical cellular contraction, such as in a striated muscle cell, is linked to cellular excitations, such as change in the sarcolemmal membrane potential
Hypertrophic cardiomyopathy (HCM)	Asymmetric hypertrophy of predominantly the left ventricle of the heart in the absence of a clinically identifiable cause such as co-morbid hypertension
Huntington's disease-like syndrome (HD-like)	A clinical entity mimicking the clinical presentation of Huntington's disease, including progressive involuntary and uncoordinated muscle movements (chorea) and subcortical dementia in a patient without an identifiable trinucleotide expansion mutation in the *HTT*-encoding huntingtin protein
Junctional membrane complex (JMC)	A complex formed by a plasma membrane and a juxtaposed intracellular membrane such as the endoplasmic reticulum or sarcoplasmic reticulum which hosts a number of proteins required for intracellular ion signaling
Purkinje cells	A type of neuron located in the cerebellum of the brain
Store-operated calcium entry (SOCE)	A cellular process to replace a depleted store of intracellular calcium, such as the sarcoplasmic reticulum, which results in net influx of calcium across the plasma membrane to replenish the store
Transient	A rapid increase in cytosolic calcium resulting from calcium-induced calcium-release
Transverse tubule (T-tubule)	A finger-like invagination of the sarcolemma of striated muscle cells which allows penetration of a membrane potential into the myocyte
Supraventricular tachycardia	An arrhythmia of the heart with rapid heart rate with an arrhythmic origin above the ventricles
Systole	Contraction of the heart or cardiac myocyte

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HIGHLIGHTS

1. Junctophilins are a conserved family of proteins found in all excitable cells
2. Junctophilins maintain subcellular architecture and calcium signaling
3. Mutations have been linked to cardiomyopathy, arrhythmia and neurodegeneration

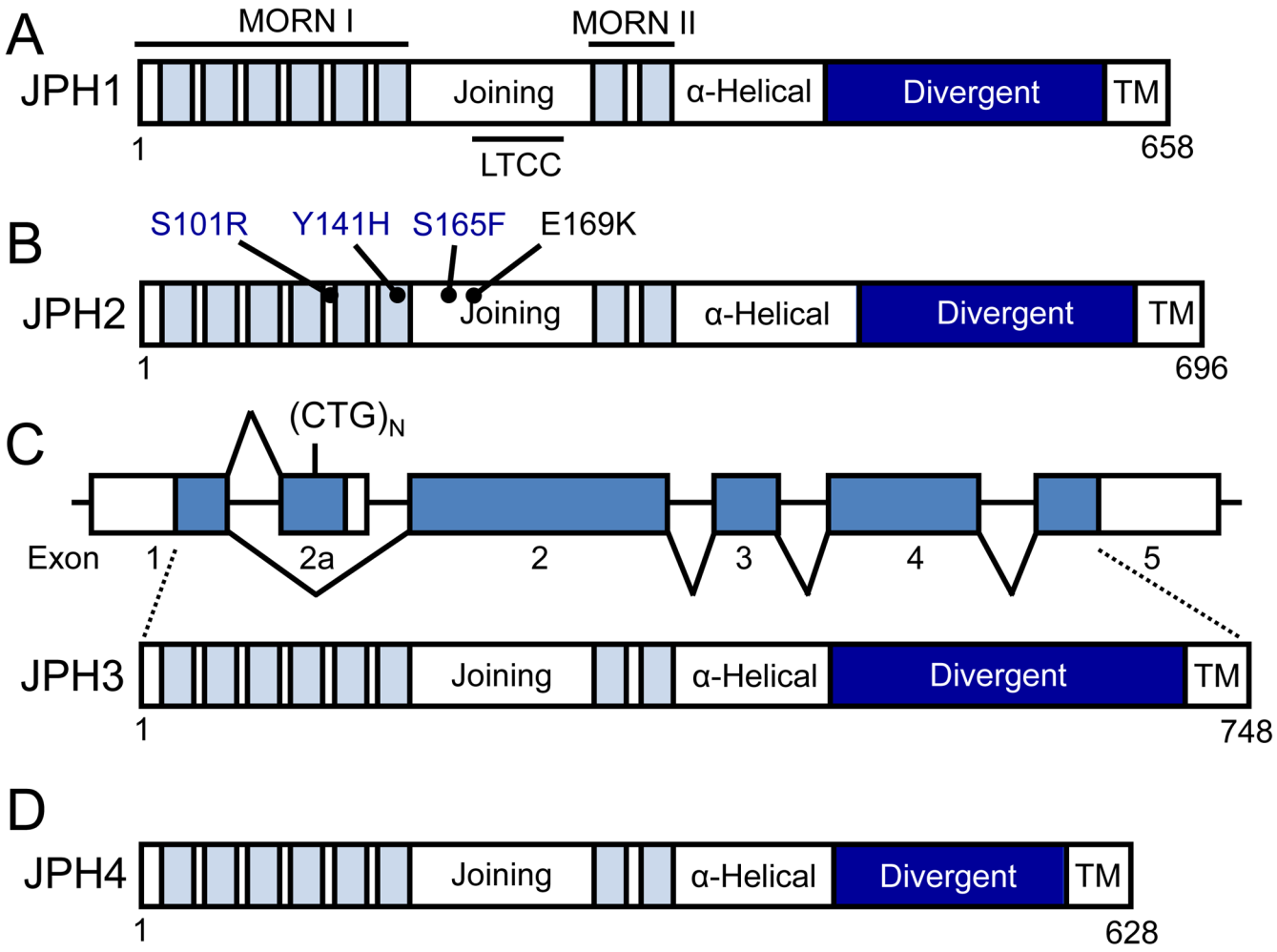

Figure 1. Protein topology of the four junctophilin isoforms expressed in humans

The JPH amino terminus has 8 MORN motifs (light blue fill) distributed across two MORN domains separated by a joining region (white fill). The carboxy terminus contains a transmembrane (TM) domain (white fill) which embeds this end of the protein into intracellular membranes such as the sarcoplasmic reticulum in striated muscle. An alpha-helical domain (white fill) and a less evolutionarily conserved divergent region (dark blue fill) join these two termini. The isoforms vary in length from 628 (JPH4) to 758 (JPH3) residues. **(A)** Protein topology of JPH1, which is found predominantly in skeletal muscle. JPH1 binds to the LTCC via a binding domain within the joining region of the protein. **(B)** Protein topology of JPH2, the major cardiac isoform. Three JPH2 mutations have been linked with the development of hypertrophic cardiomyopathy (blue text) and one to arrhythmogenesis (black text). **(C)** Schematic of the two alternatively spliced transcripts of JPH3 including the untranslated regions (white fill) and coding exons (blue fill). The full length transcript consisting of 5 exons which encode JPH3 is depicted as well as the 2 exon alternative transcript containing the CTG trinucleotide repeat expansion in the alternate exon 2 (2a). Below is the protein topology of JPH3. **(D)** Protein topology of JPH4. Abbreviations:

JPH, junctophilin; LTCC, L-type calcium channel binding domain; MORN, membrane occupation and recognition nexi; TM, transmembrane domain.

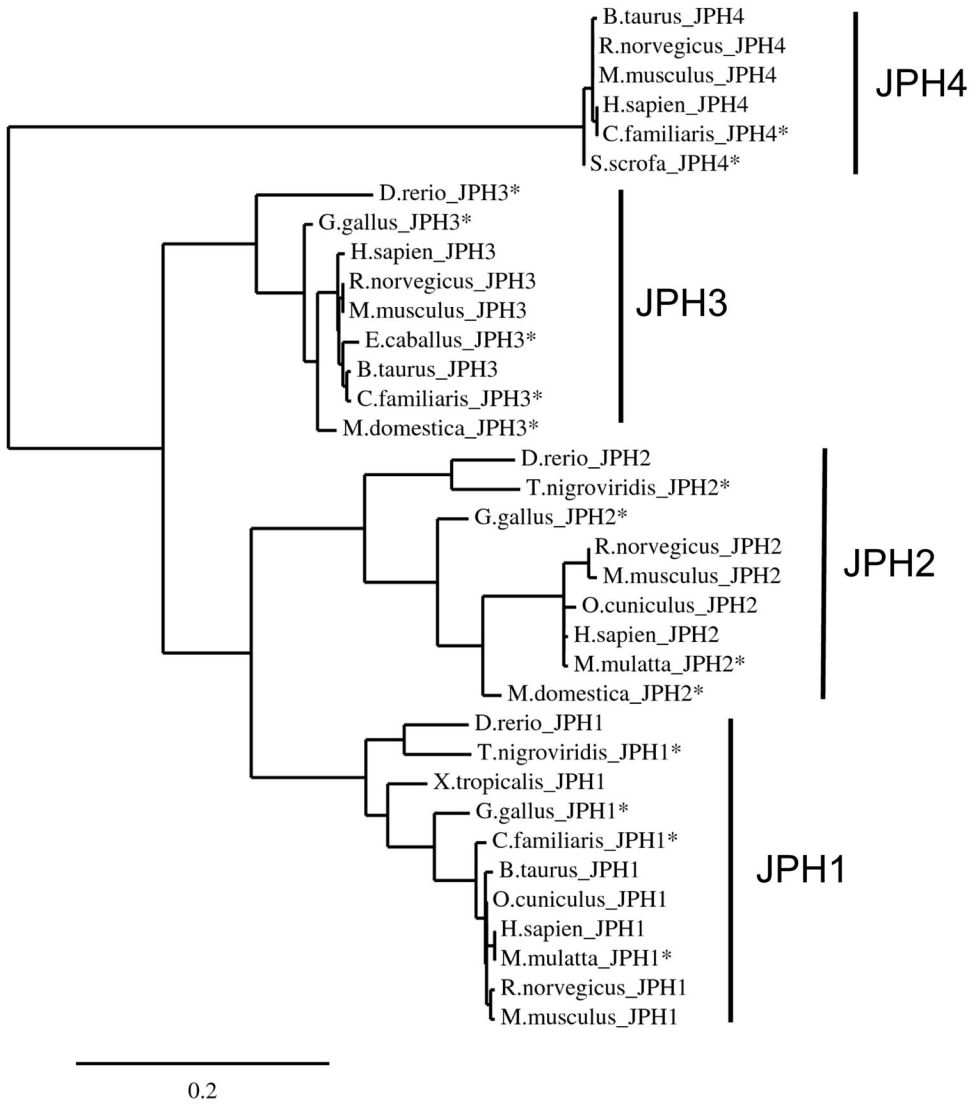

Figure 2. Junctophilin phylogenic tree

A phylogenic tree comprised of the primary sequences of 35 JPH isoforms derived from multiple species created using MUSCLE alignment and PhyML for phylogeny and TreeDyn tree rendering[4, 80–82]. Clades containing each of the isoforms are indicated. * indicate that the protein sequence is a putative JPH isoform that has not been experimentally validated. Abbreviations: JPH, junctophilin.

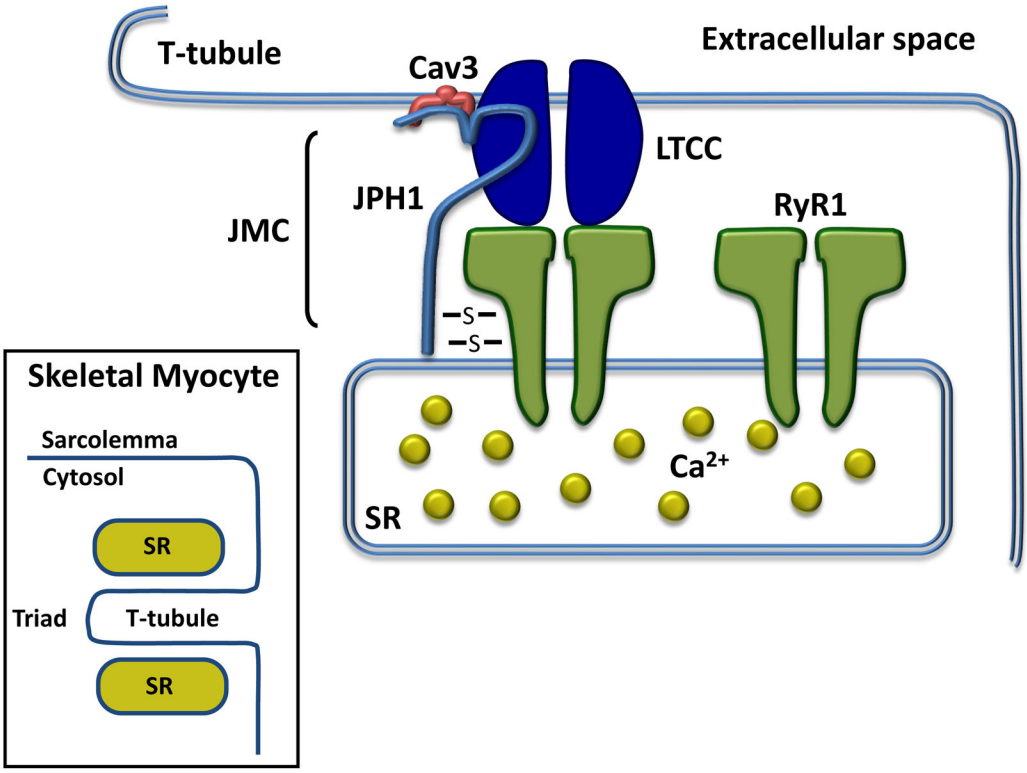

Figure 3. JPH1 and the skeletal muscle triad

The N-terminus of JPH1 localizes to the plasma membrane of the skeletal myocyte T-tubule while simultaneously approximating the sarcolemmal via a C-terminal transmembrane domain which allows it to approximate the triad subcellular membrane architecture. The N-terminal aspect of JPH1 (light blue) binds plasmalemmal-limited Cav3 (red) and the LTCC (dark blue) while the C-terminal aspect directly binds RyR1 (green) within the SR. The JPH1-RyR1 interaction is mediated by reactive thiols interactions (depicted by S) which regulate RyR1 channel gating. Together, these proteins, along with other neighboring proteins, comprise the JMC. Inset, a schematic depicting the ultrastructure of the triad within skeletal myocytes. Abbreviations: Cav3, caveolin-3; JMC, junctional membrane complex; JPH1, junctophilin-1; LTCC, L-type calcium channel; RyR1, ryanodine receptor 1; SR, sarcoplasmic reticulum.

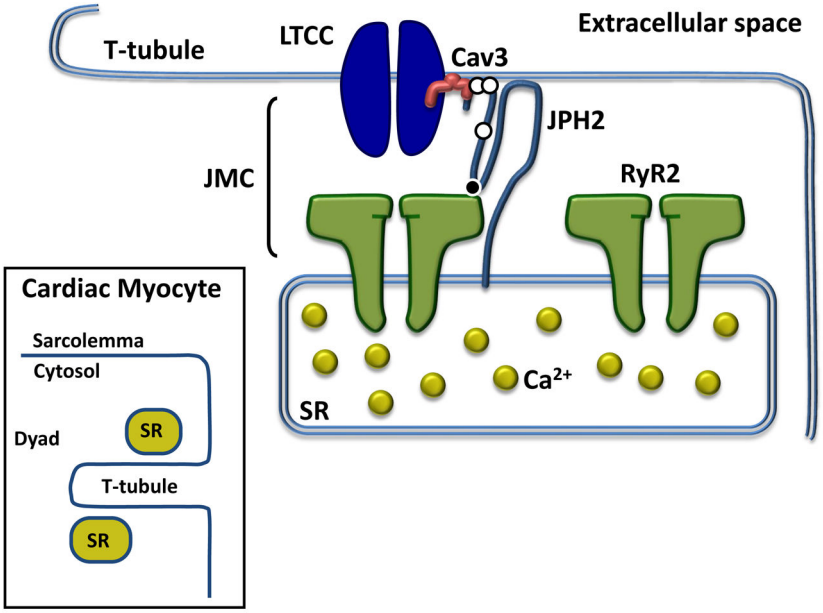

Figure 4. JPH2 and the cardiac muscle dyad

The N-terminus of JPH2 (light blue) localizes to the plasma membrane of the cardiomyocyte, binds plasmalemmal-limited Cav3 (red) and directly interacts with RyR2 (green) via the MORN motif joining region and possibly the C-terminal aspect of the protein. This interaction regulates RyR2 gating and is responsible for pathologic Ca^{2+} (yellow) release in some JPH2 mutations. Together, these proteins along with other neighboring proteins, comprise the JMC. The white circles on JPH2 indicate the position of the three mutations associated with hypertrophic cardiomyopathy while the black circle denotes the location of the mutation associated with arrhythmia. Inset, a schematic depicting the ultrastructure of the dyad within cardiac myocytes. Abbreviations: Cav3, caveolin-3; JMC, junctional membrane complex; JPH2, junctophilin-2; LTCC, L-type calcium channel; RyR2, ryanodine receptor 2; SR, sarcoplasmic reticulum.

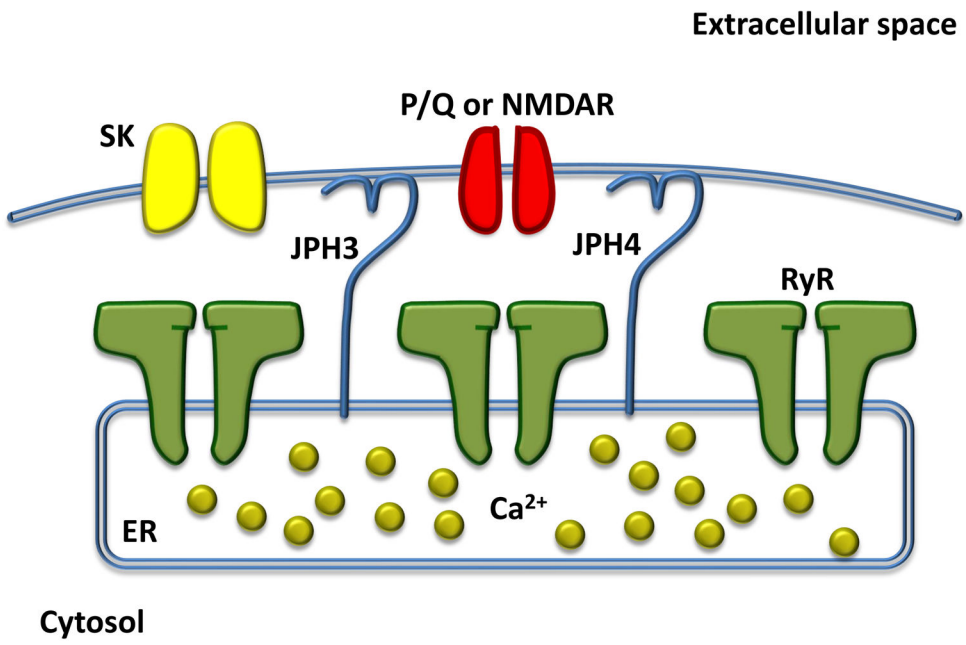

Figure 5. JPH3/4 and neuronal ultrastructure

In purkinje cells, the two JPH3 and 4 isoforms (light blue) may be responsible for maintain appropriate spacing between neuronal plasma membrane and the ER. This is critical for effective Ca^{2+} signaling from the P/Q-type channel (red), RyR (green), and SK channels (yellow). In other neuronal subtypes, such as hippocampal neurons, the NMDA receptor has been shown to mediate influx of Ca^{2+}. There is no clear evidence for direct binding of JPH3 or 4 with other members of the neuronal JMC to date. While it is hypothesized that particular RyR isoforms, including RyR1, 2, and 3, may have important roles in ER Ca^{2+} release in neuronal type-specific manner, this has yet to be clearly demonstrated Abbreviations: ER, endoplasmic reticulum; JPH3, junctophilin-3; JPH4, junctophilin-4; NMDAR, *N*-methyl *D*-aspartate glutamate receptor; P/Q, P/Q-type voltage gated channel; RyR, ryanodine receptor; SK, small-conductance Ca^{2+}-activated potassium channel.