Connexins and Disease

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Inherited or acquired alterations in the structure and function of connexin proteins have long been associated with disease. In the present work, we review current knowledge on the role of connexins in diseases associated with the heart, nervous system, cochlea, and skin, as well as cancer and pleiotropic syndromes such as oculodentodigital dysplasia (ODDD). Although incomplete by virtue of space and the extent of the topic, this review emphasizes the fact that connexin function is not only associated with gap junction channel formation. As such, both canonical and noncanonical functions of connexins are fundamental components in the pathophysiology of multiple connexin related disorders, many of them highly debilitating and life threatening. Improved understanding of connexin biology has the potential to advance our understanding of mechanisms, diagnosis, and treatment of disease.

The demand for intercellular communication arose with the evolution of multicellular organisms and in vertebrates, direct communication occurs at aggregates of intercellular channels called gap junctions. The gap junctional channels are comprised of connexins, in which each connexin has four membrane spanning domains, two extracellular loops, one intracellular loop, and intracellular amino and carboxyl termini (for a general review on connexins, see Nielsen et al. 2012). Six connexins form a con-

nexon or hemichannel that after trafficking to the plasmamembrane docks with a connexon from a neighboring cell and form an intercellular channel (see Fig. 1). Humans express 21 different connexin isoforms, each with its own distinct biophysical properties and expression pattern. Although all connexins form cell–cell channels, it has become clear that connexins also have functions that are unrelated to intercellular coupling. Undocked hemichannels can open and exchange substances or ions between

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Figure 1. Connexin structure and organization. Connexins have four transmembrane domains (TM1–4), two extracellular loops (E1–2), a cytoplasmic loop (CL), and cytoplasmic amino and carboxyl termini (NT and CT). Six connexins oligomerize to form a connexon, also known as a hemichannel, which dock with a connexon from a neighboring cell to form an intercellular gap junctional channel. Gap junctional channels form densely packed plaques that appear as a pentalaminar structure in electron microscopy (EM) (between arrows in *left* panel). Scalebar, 100 nm. (From Axelsen et al. 2013, reprinted, with permission, from the authors.)

the cytoplasm and the extracellular environment, which may exert signaling as well as pathophysiological functions (Nielsen et al. 2012). Furthermore, some connexins have channel-independent functions often associated with their ability to bind and organize cytoskeletal and signaling components (e.g., Cx43) (Leo-Macias et al. 2016a). Because connexins are expressed in most cells of the human body and exert multiple roles, their dysfunction is often associated with disease as summarized in Table 1. At this point, it seems pertinent to begin with a disclaimer. At this writing, the words “connexin” and “disease” bring close to two thousand articles in PubMed. We have attempted to reduce a large body of literature into a few pages and in doing so, we are for sure guilty of excluding many outstanding contributions that have moved the field forward. The present review touches on a limited number of diseases that may be representative of the gamut of morbidities that result from alterations in the structure and/or function of connexin proteins. We purposely chose to speak about “connexins and disease,” rather than “gap junctions and disease.” The former seeks to emphasize the fact that connexins are not only gap junction-forming proteins. There is a solid body of literature showing that membrane hemichannels can play a critical role in the ori-

gin of disease. There is also emerging literature on the role of connexins as components of multimolecular complexes, whereby loss of connexin integrity can disrupt functions seemingly foreign to those classically ascribed to either gap junctions, or connexin hemichannels. Connexins do not live in silos, isolated from other components of the cellular landscape. In the end, many years of studies on multiple “monogenic” syndromes have taught us that most diseases, including the inheritable ones, result from the balance of function within multimolecular constellations. The same becomes apparent when one reviews the topic of connexins and disease.

Cx43 IN OCULODENTODIGITAL DYSPLASIA

The library of connexin-linked diseases has grown over the past two decades to include many rare syndromes and conditions. One of the best studied connexin-linked diseases is the multisystem development disorder known as oculodentodigital dysplasia (ODDD), which is now known to be caused by nearly 80 distinct mutations in the *GJA1* gene encoding Cx43 (Paznekas et al. 2003, 2009; Laird 2014). Almost all ODDD patients have one normal *GJA1* allele

Table 1. Disease causing connexin mutations in humans

Connexin	Gene	Disease name	Phenotype	Functional alterations
Cx26	<i>GJB2</i>	Deafness PPK KID	Deafness Skin disease and deafness Skin disease and deafness	Loss of connexin expression Gain of hemichannel function Gain of hemichannel function
Cx30	<i>GJB6</i>	Deafness HED	Deafness Skin disease	Unknown Gain of hemichannel function
Cx30.3	<i>GJB4</i>	EKVP	Skin Disease	
Cx31	<i>GJB3</i>	EKVP Deafness	Skin disease Deafness	Gain of hemichannel function
Cx32	<i>GJB1</i>	CMTX1	Peripheral neuropathy and occasional CNS involvement	Loss of GJ channel function
Cx40	<i>GJA5</i>	AF	Atrial fibrillation	Loss of GJ channel function Loss of proper GJ channel trafficking
Cx43	<i>GJA1</i>	ODDD EKVP PPK	Multiple defects depending on mutation	Loss of GJ channel function Loss of proper GJ channel trafficking Gain of hemichannel function Trans-dominant effects on other connexins
Cx46	<i>GJA3</i>	Cataract	Cataract	Gain of hemichannel function Loss of hemichannel function Loss of GJ channel function
Cx47	<i>GJC2</i>	PMLD LMPH1C	Multiple CNS defects lymphedema	Loss of GJ channel function Loss of proper GJ channel trafficking Loss of hemichannel function Nonchannel effects also likely
Cx50	<i>GJA8</i>	Cataract	Cataract	Gain of hemichannel function Loss of hemichannel function Loss of GJ channel function

For review on cataract causing mutations, see Berthoud and Ngezahayo (2017), otherwise see text for references.

PPK, palmoplantar keratoderma with deafness; KID, keratitis-ichtysis-deafness syndrome; HED, hidrotic ectodermal dysplasia; EKVP, erythrokeratoderma variabilis et progressiva; CMTX1, Charcot-Marie-Tooth neuropathy X type 1; AF, atrial fibrillation; ODDD, oculodentodigital dysplasia; PMLD, Pelizaeus-Merzbacher-like disease; LMPH1C: hereditary lymphedema type IC

consistent with autosomal dominant inheritance of disease. Patients with ODDD typically have ocular defects including small eyes, dental abnormalities related to the loss of tooth enamel, soft tissue fusion of the fingers and toes, and skeletal defects particularly in craniofacial bones (Paznekas et al. 2003, 2009; Laird 2014). In addition to these common traits that are found in most ODDD patients, patients variably present with any number of comorbidities that include central nervous system (CNS) defects, bladder incontinence, skin disorders or more than a half-dozen other rare conditions (Paznekas et al. 2003, 2009; Laird 2014). Interestingly, for

reasons that remain unclear, the health status of ODDD patients tends to worsen with age presumably because of changes in the CNS (De Bock et al. 2013a). The plethora of affected organs in ODDD is somewhat predictable given that Cx43 is highly expressed in most human tissues. What was less predicted is the finding that a Cx43-rich organ, like the heart, is most often disease-free in ODDD patients, raising questions as to the mechanisms involved (Laird 2014; Kelly et al. 2015). The most prevalent explanation for how the heart is exempt from disease in ODDD is rooted in compensatory mechanisms in which other members of the 21

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connexin family are expressed in the same cardiac cells that co-express Cx43. Alternatively, and as discussed in a later section of this review, some evidence supports the premise that the heart can tolerate massive reductions in Cx43 before disease becomes evident (Flenniken et al. 2005; Kalcheva et al. 2007; Dobrowolski et al. 2008).

Over the past decade, more than two dozen Cx43 mutants have been expressed and interrogated in either reference-cells or tissue-relevant cells to assess their trafficking, assembly, and functional status (Shibayama et al. 2005; Paznekas et al. 2009; De Bock et al. 2013a; Laird 2014). These studies were combined with discoveries from three genetically modified mouse models that mirrored ODDD, to elucidate the genotype relationship of ODDD mutants to phenotype outcomes as they translate to clinical symptoms in humans (Flenniken et al. 2005; Kalcheva et al. 2007; Dobrowolski et al. 2008). Collectively, these studies revealed that *GJA1* gene mutations can cause ODDD by at least ten different mechanisms that can be subclassified as either loss-of-function or gain-of-function mutations (Kelly et al. 2015). Loss-of-function mutants include those that fail to properly traffic to the cell surface and are retained within compartments associated with protein secretion. Other mutants are still able to assemble structural gap junctions but the resulting channels are functionally dead. Gain-of-function mutants may traffic properly to the cell surface but assemble into hyperactive connexin “hemichannels.” Still other mutants in this class interact inappropriately with co-expressed connexins and show aberrant transdominant effects on a broader range of connexin channels (De Bock et al. 2013a; Laird 2014). Studies from harvested patient dermal fibroblasts have further resolved the impact of ODDD-linked mutants in a more native environment, in which the mutant and endogenous Cx43 levels are appropriately balanced in this predominantly autosomal dominant disease (Churko et al. 2011; Esseltine et al. 2015). Even with increased knowledge of the genetic and mechanistic basis of ODDD, no treatments are available for ODDD other than surgical interventions such as digit separation surgeries.

CONNEXINS IN CANCER

The year 2016 marks the 50th anniversary of Loewenstein and Kanno’s discovery that intercellular communication was absent in liver tumor cells (Loewenstein and Kanno 1966). Ensuing studies interrogating connexin and gap junction levels in a variety of tumor cell types and in vivo tumors were relatively consistent with connexins having tumor suppressive properties (Yamasaki et al. 1995; Mesnil 2002; Naus and Laird 2010). This position was supported by a diverse set of studies that reported restored growth control in tumor cells engineered to ectopically express connexins (Zhu et al. 1991; Naus et al. 1992). Other studies showed that tumor-promoting agents and cancer-causing viruses typically shutdown gap junctional intercellular communication (GJIC) in cells destined to be transformed (Crow et al. 1992). Probably the most compelling evidence linking connexins to tumorigenesis was from findings in genetically modified mice that either lacked a connexin or expressed a loss-of-function connexin mutant. For instance, mice lacking Cx32 were much more susceptible to chemical- or radiation-induced liver and/or lung tumors whereas mice expressing a Cx43 loss-of-function mutant in an ErbB2 overexpressing background had increased mammary gland tumor metastasis to the lungs (Temme et al. 1997; King and Lampe 2004a,b; Plante et al. 2011).

Over the past two decades, strategic studies have revealed that the role of connexins and gap junctions in cancer is much more complex than originally proposed. First, more exhaustive analyses of tumor types in situ and of stages of disease have revealed that some tumors are connexin-rich, and that high connexin-expression in metastatic disease may even be a negative prognostic indicator (Ito et al. 2000; Naus and Laird 2010; Zhang et al. 2015). Second, the mechanism by which connexins act in tumor cells is not limited to GJIC; in fact, several studies support a GJIC-independent action of connexins mediated through connexin “hemichannels” or the connexin interactome (Krutovskikh et al. 2000; McLachlan et al. 2006; Zhang et al. 2014). Third, the large 21 member connexin

family has potentially diverse roles in cancers as some connexins may show tumor suppressive properties in some tumor types whereas a second connexin subtype may provide no benefit (Yu et al. 2014; Yang et al. 2015). This outcome may be rooted in a naïve understanding of what passes through each connexin channel type in vivo. Lastly, epidemiological studies linking the functional status of connexins to cancer onset and disease progression is still lacking. For instance, there are hundreds-of-thousands of patients worldwide that have severely impaired Cx26 function, which typically manifests as hearing loss (Chan and Chang 2014), but there are no known reports that these patients are at increased risk for cancer in organs that are rich in Cx26, such as the liver.

Going forward, the question remains as to whether connexins are a viable target in cancer prevention or treatment. At present, although connexins are currently being targeted in clinical trials to stimulate and accelerate chronic wound repair (Becker et al. 2016), no trials targeting connexins in cancer have been initiated. In fact, although evidence suggests connexin up-regulation or functional activity may be beneficial to reduce cancer onset and primary tumor growth, complexing evidence suggest that some connexins may provide the tumor with an advantage in metastatic disease. Therefore, caution must be exercised when considering targeting connexins in cancer. Nevertheless, as genotyping patient tumors becomes more routine, it is possible to imagine where it is beneficial to target connexins in a subset of tumors as part of a combinatorial treatment program.

CONNEXINS AND NEUROLOGICAL DISORDERS

The nervous system is comprised of multiple cell types arrayed in complex interconnections. One way these various cell types interact and communicate is through gap junctions related to their functional roles (Decrock et al. 2015). Distinct connexins are expressed in unique cell types in the peripheral nervous system (PNS) and CNS; the specificity of this expression underlies the critical roles of each of these cells in

neurological functions. It is thus not surprising that the nervous system expresses up to 12 of the 21 connexin family members.

Within the mammalian PNS, gap junctions are primarily associated with the myelinating Schwann cells. Here, Cx32 forms reflexive gap junctions between the myelin lamellae connecting the Schwann cell cytoplasm with the adaxonal cell compartment inside the myelin sheath. This arrangement allows for diffusion of ions and small molecules across the apposed cell membranes comprising the myelin sheath. Cx32 forming these gap junctions thus plays a critical role in the homeostasis of myelinated axons. Indeed, this essential function of Cx32 accounts for the pathology seen in the first reported human connexin disease, namely Charcot-Marie-Tooth neuropathy X type 1 (CMTX1), a progressive peripheral neuropathy characterized by a mixed pathology of demyelination and axonal degeneration (Bergoffen et al. 1993). With more than 400 different mutations in the GJB1 gene encoding Cx32, CMTX1 is the second most common form of CMT (Kleopa and Sargiannidou 2015). Both in vitro and in vivo models of the disease indicate that most Cx32 mutations give rise to an inability of the mutant Cx32 to form functional gap junctions (Kleopa et al. 2012).

Within the mammalian CNS, various cell types express more than 10 different connexins, reflecting the complexity and diverse network of intercellular communication. These channels have distinct functions within the different cell types and their expression can change dramatically during neurodevelopment (Cina et al. 2007) and injury (Freitas-Andrade and Naus 2016). To date, three of these genes, *GJB1* (Cx32), *GJC2* (Cx47), and *GJA1* (Cx43), when mutated are associated with CNS disorders (Abrams and Scherer 2012).

The main connexins expressed in the CNS are Cx43 and Cx30 (Nagy et al. 1999); both are highly expressed in astrocytes. To a lesser extent, astrocytic Cx26 has also been reported (Nagy et al. 2001). Within these cells, gap junctions form a functional syncytium of coupled astrocytes, contributing to extracellular homeostasis through spatial buffering (Scemes and Spray 2012). Astrocytic Cx43 has been shown to un-

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derlie gap junctions functioning to spatially buffer K^+ and glutamate (Wallraff et al. 2006; Giaume et al. 2010), whereas Cx30 modulates astrocyte glutamate transport and morphology of astrocytic processes at the synaptic cleft, thereby controlling hippocampal excitatory synaptic transmission (Pannasch et al. 2014). Substantial evidence has implicated Cx43 in neuroprotection in models of ischemic and neurodegenerative conditions (Decrock et al. 2015; Freitas-Andrade and Naus 2016). In the context of human mutations of the *GJA1*, these are associated with the previously mentioned ODDD (Paznekas et al. 2003; Huang et al. 2013), a disorder that can also display CNS issues (Loddenkemper et al. 2002; Abrams and Scherer 2012) as discussed in the paragraphs above.

Oligodendrocytes express several connexins (Cx32, Cx29/31.1, Cx47), which have been shown to be necessary for proper myelination (Menichella et al. 2003), as well as potassium buffering (Menichella et al. 2006). Loss of both Cx32 and Cx47 in the CNS causes severe demyelination in mice (Menichella et al. 2003; Odermatt et al. 2003). Therefore, it was not surprising that loss-of-function mutations in *GJC2* encoding Cx47 in humans causes Pelizaeus–Merzbacher-like disease, a severe hypomyelinating leukodystrophy (Uhlenberg et al. 2004). Mutations in *GJB1*, encoding Cx32, can also result in CNS symptoms (Abrams and Scherer 2012).

Microglia constitutes the innate immune system of the CNS. The literature reports conflicting findings as to which connexins are present in microglia, but these discrepancies most likely arise as a result of different states of microglial activation (Gajardo-Gomez et al. 2016). The microglia have been reported to express Cx43 and form gap junctions (Eugenin et al. 2001), whereas other studies have not observed Cx43 immunoreactivity in microglia (Mei et al. 2010; Theodoric et al. 2012). Furthermore, Cx43 has been shown to not form gap junctions in microglia (Wasseff and Scherer 2014), but rather form hemichannels (Takeuchi et al. 2006). Expression of other connexins in microglia has also been described, including Cx32 (Dobrenis et al. 2005; Maezawa and Jin 2010),

Cx36 (Dobrenis et al. 2005), and Cx29 (Moon et al. 2010). Microglia thus appear to be very dynamic in their connexin expression profile.

The vascular cells in the CNS express several connexins (Cx37, Cx40, Cx43), which contribute to interendothelial coupling, as well as heterocellular coupling with astrocytes (De Bock et al. 2011, 2013b; Ransom and Giaume 2012). These play an important role in determining astrocyte and endothelial contributions to the blood brain barrier (BBB) (De Bock et al. 2014). This has been shown using Cx43/Cx30 double knockout mice (Ezan et al. 2012). These mice show a compromised BBB function under stress caused by increased hydrostatic vascular pressure and shear stress, swelling of astrocytic endfeet caused by edema, as well as reduced expression of aquaporin-4 and β -dystroglycan, involved in anchoring astrocyte endfeet to the perivascular basal lamina. Endothelial functions are closely regulated by junctional interactions with astrocytes; specifically important are the connexins expressed in astrocytic endfeet (Froger et al. 2010; Pannasch et al. 2011).

Depending on the maturity of the CNS, neurons can express several different connexins, including Cx36 (Belluardo et al. 2000; Venance et al. 2000; Rash et al. 2012), Cx30.2 (i.e., human Cx31.9) (Kreuzberg et al. 2008), Cx31.1 (Dere et al. 2008), Cx40 (Personius et al. 2007), and Cx45 (Li et al. 2008). This presumably reflects not only the diversity of neuronal cell types, but also varying functions in the developing CNS. Knockout (Fushiki et al. 2003; Cina et al. 2009) and knockdown (Elias et al. 2007) of Cx43 disrupts neuronal migration in the developing mouse brain, implying a possible role for gap junctions in neurodevelopmental disorders. Knockout of Cx36 in mice results in near complete loss of neuronal gap junctions in the mature CNS (Deans et al. 2001; Hormuzdi et al. 2001; Long et al. 2002; De Zeeuw et al. 2003). Surprisingly, the phenotype of these mice is rather mild, and includes loss of synchronous inhibitory activity in the hippocampus (Deans et al. 2001) and visual defects in the rod pathway (Guldenagel et al. 2001). No human mutations of the *GJD2* gene encoding Cx36 have been reported.

SKIN DISEASES AND CONNEXINS

Eleven clinically defined skin diseases with overlapping phenotypes are caused by mutations in five distinct connexin genes (Lilly et al. 2016). Six of these disorders are caused by mutations in GJB2, causing a majority of the functional research to focus on Cx26. The preponderance of evidence suggests that the skin diseases caused by connexin mutations are a result of dominant gains-of-function. In palmoplantar keratoderma with deafness (PPK), the Cx26 mutations modify the function of wild-type Cx43, resulting in leaky heteromeric hemichannels (Shuja et al. 2016). In KID keratitis-ichthyosis-deafness (KID) syndrome, Cx26 mutations form active hemichannels with increased calcium permeability, or altered regulation by calcium, voltage, and pH (Lee and White 2009; Sanchez and Verselis 2014; Sanchez et al. 2014, 2016). A detailed understanding of these subtle functional differences will be necessary to develop mechanistic-based therapies for connexin-related skin diseases.

The five connexins linked to skin disease have overlapping patterns of expression. In normal adult human skin, Cx26 is restricted to basal keratinocytes in palmoplantar skin, whereas Cx43 is expressed in all epidermal layers. Cx26 and Cx43 are also present in sweat glands and hair follicles. In contrast, Cx30, Cx30.3, and Cx31 are only expressed in the upper differentiated epidermal layers (Salomon et al. 1994; Di et al. 2001). A large induction of Cx26 expression is observed across the epidermis in human skin after wounding, or other pathological and experimental changes in skin thickness (Rivas et al. 1997; Labarthe et al. 1998; Lucke et al. 1999; Rouan et al. 2001), which could have profound consequences on pathology caused by dominant gain-of-function mechanisms following Cx26 mutation.

Recent studies have begun to elucidate how increased hemichannel activity could contribute to changes in epidermal homeostasis associated with the connexin genodermatoses. Hemichannels form aqueous pores, allowing inorganic ions and metabolites to pass between the cytoplasm and extracellular space. Electrophysiolog-

ical studies using cysteine scanning revealed that many of the residues mutated in KID syndrome lined the hemichannel pore, and that calcium regulation of the hemichannels differed. Some of the KID mutations become insensitive to inhibition of hemichannel activity by extracellular Ca^{2+} , whereas others showed only modest impairment in Ca^{2+} gating. However, mutant hemichannels with modestly impaired gating showed a substantially increased permeability to Ca^{2+} (Sanchez et al. 2010, 2013, 2016). Thus, KID syndrome mutations all show hemichannel gain-of-function that produce epidermal pathology, but through different mechanisms, potentially explaining the phenotypic heterogeneity among patients carrying different mutations. The epidermis maintains an extracellular calcium gradient, with the highest levels in the granular layer and an abrupt reduction in the cornified layer, and calcium is a key regulator of keratinocyte differentiation (Elsholz et al. 2014). The altered Ca^{2+} permeation properties of KID hemichannels would almost certainly disrupt this gradient and affect keratinocyte differentiation. Additional studies using mouse models of KID syndrome have provided further support for mutant hemichannels disrupting calcium homeostasis in vivo. Transgenic mice expressing KID develop epidermal pathology, and increased hemichannel activity was detected in primary keratinocytes isolated from KID lesions (Mese et al. 2011; Schutz et al. 2011). When the epidermal calcium gradient was examined in the skin of these mice, it was found to be elevated in both the intra- and extracellular compartments of the cornified layer. This change in Ca^{2+} homeostasis was associated with defects in the epidermal water barrier and altered lipid secretion in the affected skin (Bosen et al. 2015).

Increased hemichannel activity has been compellingly linked to specific Cx26 mutations causing KID syndrome, raising the question as to whether there may be a generalized role for dysregulated hemichannels in some of the other connexin skin disorders. Evidence that altered hemichannel function may also play a role in other connexin genodermatoses comes from studies of Cx30 mutations associated with hidrotic ectodermal dysplasia (HED). Expression

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of these mutants in vitro caused cell death that was correlated with large voltage-activated currents in cells expressing mutant proteins. In addition, cells expressing the mutant connexins had a much higher rate of ATP leakage than controls, suggesting that hemichannel-mediated ATP release may contribute to HED syndrome pathology (Essenfelder et al. 2004). In a similar fashion, Cx31 mutations linked to erythrokeratoderma variabilis et progressiva (EKVP) and Cx43 mutations causing keratoderma-hypotrichosis-leukonychia totalis syndrome (KHLT) were both shown to result in increased hemichannel activity, Ca^{2+} , or ATP leakage, and cell death when expressed in transfected cells (Chi et al. 2012; Wang et al. 2015).

A growing body of evidence suggests that many, if not a majority, of the connexin mutations associated with distinct skin diseases consistently acquire unique gain-of-function activities. So far, the most prominent mechanism is the formation of dysregulated hemichannels that either increase ATP leakage, or disrupt Ca^{2+} homeostasis within keratinocytes and in the extracellular gradient. Further study of disease-causing mutations coupled with additional analysis of transgenic mice expressing them in native epidermis will continue to provide new mechanistic insight into the pathophysiology of the connexin genodermatoses.

CONNEXINS AND DEAFNESS

One of the most common causes of autosomal recessive, nonsyndromic, sensorineural deafness is mutation of the *GJB2* gene encoding the Cx26 gap junction (GJ) protein. Remarkably, although mutations in more than 90 genes have been identified that are associated with deafness, Cx26 mutations account for as much as 50% of hearing loss characterized as severe to profound. Mutations in the *GJB6* gene encoding Cx30 also cause deafness, but are much less frequent.

In addition to forming GJ channels that signal between cells, Cxs can function as undocked hemichannels that signal across the plasma membrane, as outlined in the introduction. In the cochlea, Cx26 and Cx30 are expressed in the supporting cells of the organ of Corti surround-

ing the sensory hair cells as well as in the lateral wall containing the stria vascularis (Lautermann et al. 1998; Ahmad et al. 2003; Forge et al. 2003; Zhao and Yu 2006; Liu and Zhao 2008). The stria vascularis is responsible for producing endolymph and generating the endocochlear potential, EP, which is critical for proper signal transduction in hair cells. Both Cxs show widely overlapping patterns of expression and an abundance of GJs indicative of extensive interconnected cellular networks. There is also surface expression of Cx26 in basolateral and apical regions of supporting cells in regions devoid of cell-cell contacts, indicating the presence of hemichannels that can communicate with perilymphatic and endolymphatic compartments (Majumder et al. 2010).

The majority of nonsyndromic Cx26 mutations are deletions, truncations, and frameshifts indicating that loss of GJ and/or hemichannel function results in hearing loss. However, there are a number of dominant, missense mutations that produce functional channels and result in syndromes in which hearing loss is accompanied by severe skin disorders, such as KID syndrome (reviewed above) (Lee and White 2009; Xu and Nicholson 2013).

Studies of Cx26 knockout (KO) mice and transgenics with dominant-negative activity are in general agreement that loss of Cx26 channel function, even when restricted to supporting cells in the sensory epithelium, produces deafness and leads to eventual death of supporting cells and hair cells (Cohen-Salmon et al. 2002; Kudo et al. 2003; Maeda et al. 2007; Sun et al. 2009). Reports of hearing loss before evidence of cell death or rundown of the EP have suggested a role for Cx26 in the developing cochlea (Wang et al. 2009; Liang et al. 2012; Chen et al. 2014). Knockout of Cx30 in mice was also reported to cause deafness with rundown of the EP (Teubner et al. 2003; Schutz et al. 2010). However, expression of Cx26 was shown to decrease in the cochleae of Cx30 null mice (Ahmad et al. 2007; Ortolano et al. 2008) and when Cx26 expression was preserved or increased in Cx30-null mice, hearing loss was prevented (Ahmad et al. 2007; Boulay et al. 2013). Increasing Cx30 expression in Cx26 null mice did not prevent

hearing loss (Qu et al. 2012). Thus, the presence of Cx26, but not Cx30 channels in the sensory epithelium is crucial for hearing, whereas either Cx26 or Cx30 in the stria vascularis appears capable of maintaining hearing.

Mechanistically, although GJs in the stria vascularis are undoubtedly important for K⁺ transport and maintenance of the EP, a role for GJs in K⁺ transport within the sensory epithelium is not supported (Cohen-Salmon et al. 2002; Kudo et al. 2003; Wangemann 2006; Zdebik et al. 2009; Patuzzi 2011). Rather, GJs likely communicate nutrients and signaling molecules, as shown for glucose and inositol triphosphate (IP₃), which presumably maintain sensory epithelial integrity and aid in proper hair cell function (Beltramello et al. 2005; Zhang et al. 2005; Chang et al. 2008, 2009).

Hearing loss in syndromic deafness likely differs from the nonsyndromic form with mutant hemichannels that are overactive and/or show altered Ca²⁺ permeability leading to cell dysfunction and death (Stong et al. 2006; Gerido et al. 2007; Mese et al. 2008; Lee et al. 2009; Sanchez et al. 2010, 2013, 2014, 2016; Terrinoni et al. 2010; Levit et al. 2011, 2011; Mhaske et al. 2013; Garcia et al. 2015). For a number of syndromic deafness mutants, overactive hemichannels have been shown to be a consequence of weakened inhibition by extracellular divalent cations, particularly Ca²⁺ and by pH (Gerido et al. 2007; Lee et al. 2009; Sanchez et al. 2010, 2013, 2014, 2016; Levit et al. 2011; Mhaske et al. 2013). These mutant hemichannels can cause excessive membrane depolarization and initiate damaging membrane signaling. Altered permeability can also lead to damaging membrane signaling as suggested for the G45E KID syndrome mutation that is characterized by enhanced Ca²⁺ permeability (Sanchez et al. 2010). Patients carrying this mutation show profound deafness corroborated by brainstem audiometry (Gilliam and Williams 2002; Janecke et al. 2005; Griffith et al. 2006). An autopsy of a G45E patient showed widespread vestibulo-cochlear dysplasia in the organ of Corti with no evidence of a developed sensory epithelium (Griffith et al. 2006), plausibly explained by excessive Ca²⁺ entry and cell death during cochlear development. Corre-

lates between patient phenotype and aberrant hemichannel function are emerging and their identification should shed light on the roles connexins play in cochlear function and provide avenues for treatment.

CONNEXINS AND HEART DISEASE

Cardiac cells mainly contain the connexins Cx43>Cx40>Cx45>Cx31.9 (please note that human Cx31.9 is the homolog of mouse Cx30.2) with Cx43 being the overall dominating and the sole gap junction protein in the ventricular myocardium. Gap junctions have been suggested to play a role in a number of cardiac pathologies, causing both electrical and structural disturbances.

Early observations showed that acute ischemia caused an increase in tissue resistivity, most likely by uncoupling of gap junctions (Wojtczak 1979; Kleber et al. 1987), and uncoupling correlated with the appearance of arrhythmia (Smith et al. 1995). Acute uncoupling occurs with a time course similar to that of intracellular acidosis and increased calcium (Carmeliet 1999), conditions that are known to close Cx43 gap junction channels (Burt 1987; Firek and Weingart 1995; Morley et al. 1996); but in addition to direct chemical gating, changes in Cx43 phosphorylation (Beardslee et al. 2000; Axelsen et al. 2006; Lampe et al. 2006) and lipid metabolites (Fluri et al. 1990; Hofgaard et al. 2008; Mollerup et al. 2011) may also contribute. Evidence suggests that uncoupling makes hearts more prone to reentrant arrhythmias, based on studies using pharmacological uncoupling (Ohara et al. 2002), prevention of uncoupling (Xing et al. 2003; Lewandowski et al. 2008), and genetic ablation of Cx43 (Danik et al. 2004). At least in some studies, ablation of Cx40 lowers atrial conduction velocity and increases atrial fibrillation (AF) risk (Hagendorff et al. 1999; Verheule et al. 1999; Bagwe et al. 2005), aligning well with studies linking Cx40 mutations and AF in humans (Gollob et al. 2006), of which at least one mutation replicates the phenotype when expressed in mice (Lubkemeier et al. 2013a). More recently, a mutation in Cx43 has also been linked to AF (Sinner et al. 2014).

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Gap junctions are not only involved in electrical abnormalities but also in determining infarct size after ischemia. Uncoupling was originally suggested to have beneficial effects by preventing spread of tissue damage. In support of this notion, chemical uncoupling reduces infarct size in animal models as first described by Garcia-Dorado and coworkers (1997). Although based on rather nonspecific inhibitors, these observations were initially a strong argument against the development of anti-arrhythmic therapies aimed at keeping gap junctions open. Using anti-arrhythmic peptides (rotigaptide and danegaptide) to keep gap junctions open, however, decreased rather than increased infarct size (Haugan et al. 2006; Hennen et al. 2006; Skyschally et al. 2013). The issue of the relation between gap junction availability and cardiac homeostasis extends not only to whether a gap junction channel is present, but also to whether those channels can be regulated by the intracellular milieu. For example, infarct size was increased in mice expressing carboxy-terminal truncated Cx43, which does not respond to pH and calcium induced gating (Maass et al. 2009). Furthermore, reducing Cx43 to 50% by genetic ablation did not alter infarct size in mice (Schwanke et al. 2002). In contrast, preconditioning was lost in the Cx43^{+/-} mice (Schwanke et al. 2002) independent of an effect on intercellular coupling (Li et al. 2004), an observation that led to the somewhat surprising discovery that Cx43 locates to mitochondria and protects them from ischemic damage (Boengler et al. 2005). This observation adds to a growing list of coupling-independent effects of connexins, as discussed below.

Following ischemia, gap junctions distribute heterogeneously with lateralization in the infarct border zone and these areas of disarray also display reentrant arrhythmias on local stimulation (Peters et al. 1997). Similar disarray is observed under several pathological conditions including heart failure (Kostin et al. 2003), systemic (Kostin et al. 2004), and pulmonary (Chkourko et al. 2012) hypertension; and although it is often assumed that lateralized gap junctions are not functional, a study suggests that they are (Cabo et al. 2006). The issue of lateralization of con-

nexins and their impact on cardiac electrophysiology remains unclear.

Recent evidence shows that connexins have functions unrelated to intercellular coupling, and likely consequent to the fact that connexins are components of a protein interacting network (a “connexome”) that includes molecules classically defined as belonging to mechanical junctions and to the voltage-gated sodium channel complex (Agullo-Pascual et al. 2014a,b). As a component of the connexome, Cx43 actually helps organize the microtubular network (Francis et al. 2011; Agullo-Pascual et al. 2013a,b). In fact, mislocalization of Cx43 can alter the trafficking of other proteins; along those lines, knocking Cx43 out in mice reduces the cardiac sodium current and contributes to arrhythmia susceptibility (Jansen et al. 2012). Knockdown of Cx43, removal of the carboxyl terminus, or of the tubulin binding domain also reduced the surface expression of NaV1.5 in HL-1 cells (Agullo-Pascual et al. 2014b). Similarly, removal of the PDZ-binding domain of Cx43 led to decreased sodium current and lethal arrhythmias (Lubkemeier et al. 2013b). Conversely, treating hearts injured by cryo-ablation with a peptide containing the carboxy-terminal PDZ-binding domain improved Cx43 localization and reduced arrhythmia burden (O’Quinn et al. 2011). Electrophysiological recordings indicated that conduction velocity was increased, but to what extent this finding could be explained by preservation of sodium current, was not tested.

The role of the gap junction plaque on action potential propagation may extend beyond the formation of the gap junction channel per se. In recent years, the concept of ephaptic or electrical field-mediated propagation has re-emerged, placing the ephaptic phase as peripheral to the gap junction (in the “perinexus” as defined by the Gourdie laboratory (Rhett et al. 2011)). Ephaptic coupling occurs when influx of sodium leads to a local extracellular negative potential, which effectively depolarizes the opposing membrane. Conditions for this to occur are predominantly a large and dense sodium current, and closely positioned membranes (Sperelakis 2002). These conditions are possibly met at the intercalated disc. In addition to the

perinexus (Rhett et al. 2012), sodium channels are confronted against each other at the adhesion/excitability nodes, “mini-nodes of Ranvier” where clusters of N-cadherin organize sodium channels into dense clusters (Leo-Macias et al. 2016b). Evidence for ephaptic coupling includes the finding that extracellular edema, contrary to expectation from cable theory, decreases rather than increases CV (Veeraraghavan et al. 2012). Ephaptic theory may also explain the paradox that in heterozygous Cx43^{+/-} mice, reduction of connexin abundance does not associate with changes in conduction velocity (Morley et al. 1999; Stein et al. 2009).

CONCLUDING REMARKS

Limitations in pharmacological tools and in methods for studying connexin function in vivo initially made it difficult to directly link connexin function to disease. It is ~30 years since the identification of the genes coding for gap junction proteins, which enabled us to link connexins to specific functions by the discovery of disease-causing mutations. In some cases, mutations confirmed our understanding of connexins as gap junctional channels that provide intercellular coupling, but in other cases, new noncanonical functions have emerged. The combined genetic and functional investigations have led to enormous leaps in understanding not only the function of these proteins but also their role in inherited and in acquired diseases. In both cases, we have learned that rarely is a disease caused by a single molecule, without the complicity of its neighbors. Phenotypes are the reflection of a constellation of molecules that work together, or fail to do so. Among the many challenges ahead is to gain a better understanding of how these constellations are formed, and how connexins operate within them so that we can better connect the two poles of a spectrum: connexin and disease.

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