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Connexins in Cardiovascular and Neurovascular Health and Disease: Pharmacological Implications

Luc Leybaert, Paul D. Lampe, Stefan Dhein, Brenda R. Kwak, Peter Ferdinandy, Eric C. Beyer, Dale W. Laird, Christian C. Naus, Colin R. Green, and Rainer Schulz

Physiology Group, Department of Basic Medical Sciences, Faculty of Medicine and Health Sciences, Ghent University, Ghent, Belgium (L.L.); Translational Research Program, Fred Hutchinson Cancer Research Center, Seattle, Washington (P.D.L.); Institute for Pharmacology, University of Leipzig, Leipzig, Germany (S.D.); Department of Pathology and Immunology, Department of Medical Specialization–Cardiology, University of Geneva, Geneva, Switzerland (B.R.K.); Department of Pharmacology and Pharmacotherapy, Semmelweis University, Budapest, Hungary (P.F.); Pharmahungary Group, Szeged, Hungary (P.F.); Department of Pediatrics, University of Chicago, Chicago, Illinois (E.C.B.); Department of Anatomy and Cell Biology, University of Western Ontario, Dental Science Building, London, Ontario, Canada (D.W.L.); Cellular and Physiological Sciences, Faculty of Medicine, The University of British Columbia, Vancouver, British Columbia, Canada (C.C.N.); Department of Ophthalmology and The New Zealand National Eye Centre, Faculty of Medical and Health Sciences, University of Auckland, Auckland, New Zealand (C.R.G.); and Physiologisches Institut, Justus-Liebig-Universität Giessen, Giessen, Germany (R.S.)

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P. Ferdinandy is the founder and CEO of Pharmahungary, a group of R&D companies.

Address correspondence to: Luc Leybaert, Department of Basic Medical Sciences, Faculty of Medicine and Health Sciences, Ghent University, De Pintelaan 185, B9000 Ghent, Belgium. E-mail: Luc.Leybaert@UGent.be

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ABBREVIATIONS: AF, atrial fibrillation; APD, action potential duration; $[Ca^{2+}]_e$, extracellular Ca^{2+} concentration; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CK, casein kinase; CL, cytoplasmic loop; CNS, central nervous system; CT, C-terminal tail; DCM, dilated cardiomyopathy; EL, extracellular loop; FGF, fibroblast gap factor; GJ, gap junction; γ, single channel conductance; HC, hemichannel; HDAC, histone deacetylase; ICM, ischemic cardiomyopathy; ID, intercalated disk; IL, interleukin; IP_3, inositol, 1,4,5 trisphosphate; IPC, ischemic preconditioning; KO, knockout; LDL, low-density lipoprotein; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; miRNA, microRNA; MS, mass spectrometry; MM, molecular weight; NO, nitric oxide; NT, N-terminal tail; ODDD, oculodentodigital dysplasia; PKC, protein kinase C; pMCAO, permanent middle cerebral artery occlusion; ROS, reactive oxygen species; SH3, Src homology domain 3; SNP, single nucleotide polymorphism; TM, transmembrane domain; TNF-α, tumor necrosis factor-α; VF, ventricular fibrillation; V_j, junctional potential difference; V_m, membrane potential; ZO-1, zonula occludens 1; ZONAB, ZO-1–associated nucleic acid binding protein.

Abstract——**Connexins are ubiquitous channel forming proteins that assemble as plasma membrane hemichannels and as intercellular gap junction channels that directly connect cells. In the heart, gap junction channels electrically connect myocytes and specialized conductive tissues to coordinate the atrial and ventricular contraction/relaxation cycles and pump function. In blood vessels, these channels facilitate long-distance endothelial cell communication, synchronize smooth muscle cell contraction, and support endothelial-smooth muscle cell communication. In the central nervous system they form cellular syncytia and coordinate neural function. Gap junction channels are normally open and hemichannels are normally closed, but pathologic conditions may restrict gap junction communication and promote hemichannel opening, thereby disturbing a delicate cellular communication balance. Until recently, most connexin-targeting agents exhibited little specificity and several off-target effects. Recent work with peptide-based approaches has demonstrated improved specificity and opened avenues for a more rational approach toward independently modulating the function of gap junctions and hemichannels. We here review the role of connexins and their channels in cardiovascular and neurovascular health and disease, focusing on crucial regulatory aspects and identification of potential targets to modify their function. We conclude that peptide-based investigations have raised several new opportunities for interfering with connexins and their channels that may soon allow preservation of gap junction communication, inhibition of hemichannel opening, and mitigation of inflammatory signaling.**

I. Introduction

Connexins are ubiquitous integral membrane proteins present in almost all cells of the body. They are strongly expressed in major organs such as the heart, brain, and liver, as well as in endothelial and smooth muscle cells of blood vessels. Their main function is to facilitate cell-cell communication and they do so in the most direct way possible, by forming channels called gap junctions (GJs) that connect the cytoplasm of cells. This short route connection serves as a powerful coordinator of cell function in complex tissues like heart and brain; it also permits efficient long-distance communication along rows of GJ-connected cells, as e.g., in the His-Purkinje conduction system in the heart or in endothelial cells of the blood vessel wall to transmit upstream vasodilatory messages (de Wit and Griffith, 2010). In electrically excitable cells like cardiac myocytes, GJ channels facilitate electrical coupling by allowing cell-to-cell passage of ions. Action potentials spread from one cell to another via GJs that are mainly localized at the cell poles in the plicate and interplicate regions of the intercalated disk (ID) (Spach and Heidlage, 1992). Isolated individual cardiomyocytes do not communicate, but when manipulated into close contact with each other, they start to communicate electrically within a couple of minutes via newly established GJ channels (Weingart and Maurer, 1988). The importance of connexins is clear from mouse knockout studies of the major cardiovascular connexins, which yield a nonviable phenotype for $Cx26^{-/-}$, $Cx37^{-/-}$–$Cx40^{-/-}$ double knockouts (KOs), $Cx43^{-/-}$, and $Cx45^{-/-}$ (reviewed in Simon et al., 1998; Söhl and Willecke, 2004). An example illustrating the importance of connexins in the human body concerns inherited mutations in the *GJB2* gene that codes for Cx26, which cause congenital sensorineural deafness that has a prevalence estimated in the order of 1:5000 births (Chan and Chang, 2014;

Esseltine and Laird, 2016). Other examples include polymorphisms of *GJA4* (Cx37), which are linked to vascular disease, mutations of *GJA5* (Cx40), which are known to predispose for atrial fibrillation and *GJA1* mutations (Cx43), which are generally not associated with a cardiac phenotype (Pfenniger et al., 2011; Delmar and Makita, 2012; Molica et al., 2014) but may lead to oculodentodigital dysplasia (ODDD), a rare primarily autosomal dominant clinical syndrome characterized by multiple malformations. An overview of connexin genes and chromosome locations can be found in Table 1 of Söhl and Willecke (2004); for the distribution of the various connexins in organs and tissues see Table 2 in Laird (2006).

GJs were discovered half a century ago (Revel and Karnovsky, 1967; Brightman and Reese, 1969), and their connexin building blocks were discovered more than 40 years ago (Goodenough, 1974). GJs are dodecameric channels formed by the interaction of two opposed hexameric hemichannels (HCs), also called connexons. Molecular cloning studies have established that connexins form a family of related proteins (Beyer et al., 1990). Twenty-one connexin genes have been identified in the human genome and 20 in the murine genome, which encode proteins with a molecular mass (MM) that ranges from 23 to 62 kDa (Söhl and Willecke, 2004; Beyer and Berthoud, 2009). Connexins are named according to their MM; they have a tetraspan membrane topology, with four transmembrane (TM) domains, two extracellular loops (EL1, EL2), a cytoplasmic loop (CL), and their N- and C-terminal tails (NT and CT) located inside the cell (Fig. 1). The channels formed by the different connexins often also differ in their gating properties, conductances, and permeabilities to various ions and molecules. In general, GJ channels have a pore diameter in the 10–20 Å range and grant passage not only to atomic ions such as K^+, Na^+, or Ca^{2+}, but also to metabolic molecules with a MM below ~1.5 kDa (assuming an approximate spherical shape) like ATP, glucose, ascorbic acid, or glutathione,

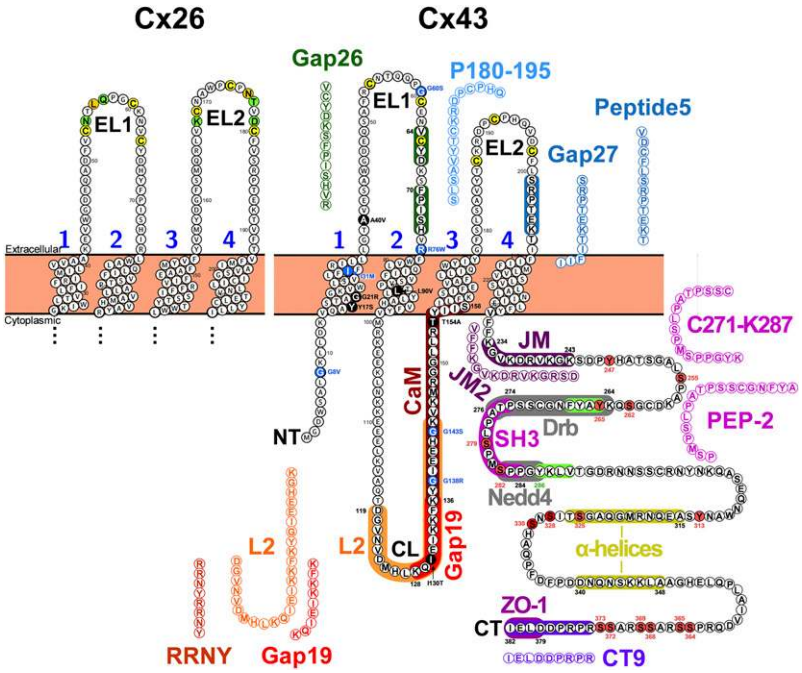

Fig. 1. Topology of human Cx26 and Cx43 indicating crucial domains as well as peptides that affect protein and channel functions. (Left) Illustrates the extracellular loops (EL1, EL2) of Cx26 (intracellular protein parts not shown), indicating the position of highly conserved Cys residues (three per loop, yellow-filled circles) that act to stabilize the loops. Crucial residues involved in interactions between the loops upon docking of two opposed hemichannels are also indicated; for EL1, Asn-54 (green-filled circle) forms a hydrogen bond with Leu-56 of EL1 from the opposite connexin (orange), whereas Gln-57 (green) forms a hydrogen bond with Gln-57 from the opposite connexin. For EL2, salt bridges are formed between Lys-168, Asp-179, and Thr-177 from one side (green) with Asn-176 from the opposite connexin (orange). (Right) Illustrates the Cx43 topology indicating the location of conserved EL1/EL2 Cys residues (yellow). Several important domains are illustrated including the VCYD and FPISH motifs on EL1, SRPTEK on EL2 and the L2, Gap19, and CaM (calmodulin interaction site) sequences on the cytoplasmic loop (CL). Domains on the C-terminal tail (CT) include the tubulin-binding JM domain (juxtamembrane, crucial for microtubule binding), the drebrin-binding domain (Drb; crucial domain indicated; links Cx43 to F-actin), the Nedd4 domain (ubiquitin ligase), SH3 domain, and the ZO-1–binding domain (links Cx43 to F-actin). Peptides mimicking some of these domains are illustrated in the color of the corresponding domain. RRNY peptide (RRNYRRNY) is not a mimetic peptide; like L2 and Gap19, it interacts with the Cx43 CT and prevents GJ closure while inhibiting HC opening. JM2 peptide is a mouse version that differs in residue 243 from the human. The two yellow-marked CT sequences (315–326 and 340–348) are α-helical domains, whereas the rest of the CT is intrinsically disordered. The light green-marked domains are important Tyr-based sorting sequences involved in Cx43/GJ internalization (Y^{265}AYF, Y^{286}KLV). Blue-filled circles with white amino acid letter codes indicate mutations characterized by increased HC function relative to the function of GJs. Black-filled circles with white letter codes indicate ODDD mutations with reduced GJ and HC function. Red-filled circles with black Tyr or Ser indicate major phosphorylation sites, including CK1-targeted Ser-325,328,330, MAPK-targeted Ser-255,262,279,282, PKC-targeted Ser-368, Akt/PKB-targeted Ser-369,373, and Src/Tyk2-targeted Tyr-247,265,313. Ser-364,365 is phosphorylated by PKA but in an indirect manner involving other kinases. A detailed account on the role of the various amino acids and domains illustrated here can be found in *sections II* and *III*.

and second messengers such as cAMP, cGMP, or inositol trisphosphate (IP_3) (Alexander and Goldberg, 2003; Saez et al., 2003; Li et al., 2012a). Recent evidence indicates that siRNA and miRNA may also pass through GJs, most likely as a rod-shaped molecule linearly permeating the pore with its smallest dimension (Brink et al., 2012). As a result of the passage of multiple substances, which can exert beneficial but also toxic effects, GJs may also be involved in communicating and spreading harmful messages. Two marked examples of such inadequate signaling include the propagation of inflammation along the blood vessel wall (Parthasarathi et al., 2006) and bystander cell death propagation in ischemic cardiomyocytes and brain cells (Contreras et al., 2004; García-Dorado et al., 2004; Decrock et al., 2009b). GJs can close and uncouple cells in response to various conditions, either as a result of mutations or activation of signaling cascades. As illustrated above, several connexin mutations with impact on the cardiovascular system or the brain have been characterized (reviewed in Pfenniger et al., 2011; Abrams and Scherer, 2012; Delmar and Makita, 2012; De Bock et al., 2013a; Molica et al., 2014). GJs also typically close under ischemic conditions, as a result of intracellular acidification and increased cytoplasmic Ca^{2+} as forefront signals (Kleber, 1992; Anderson et al., 2003; Dhein, 2006a; Evans, 2015; Moore and O'Brien, 2015). Additionally, GJs are modulated in various ways by posttranslational modifications. In principle, GJs can be targeted by pharmacological inhibitors to counteract their contribution to bystander cell death, but in heart as well as in brain, the success of such an approach is not guaranteed because it will compromise the physiologic roles of GJs, i.e., impulse propagation in the heart or substrate delivery through the GJ-connected astrocytic network (Rouach et al., 2008), which also contributes, albeit partially, to spatially buffering K^+ away from zones of high neuronal activity (Wallraff et al., 2006). To preserve physiologic functions, GJs may be targeted to correct or prevent uncoupling. In stroke and spinal cord injury, knockout of specific connexins often gives different outcomes compared with pharmacological GJ inhibition, which may result from channel-independent connexin functions that remain operational in the latter case while absent in knockout. Another player that has entered the field and attracted considerable interest as a novel pharmacological target are the connexin HCs.

Although GJs have been investigated for over half a century, the interest in possible functions of HCs has a more recent origin. HCs were supposed to be closed until they interact and form a GJ channel at which point they open. It is only since the early 1990s that it was realized that HCs could open without necessarily forming a GJ, resulting in a conduit that communicates with the extracellular space, not with a neighbor cell (Paul et al., 1991). It is now clear that the spectrum of connexin functions encompasses actions of the connexins themselves (channel-independent functions), functions related to GJs, and functions related to HC opening. GJs have well established physiologic roles but they may additionally exert pathologic effects, e.g., by contributing to bystander cell death (Decrock et al., 2009b, 2017). By contrast, HCs have mainly been implicated in pathologic contexts, although it is not clear whether they have any physiologic function. HCs have roughly the same upper limit of ~1.5 kDa for passing substances but passage is predicted to be easier as the channel is half as long as a GJ channel (the conductance is twice as large for a HC compared with a GJ channel). HCs allow the free passage of Na^+ and K^+, which may lead to cell swelling, and facilitate Ca^{2+} entry (Schalper et al., 2010; Fiori et al., 2012), which may cause cellular Ca^{2+} overload and promote the escape of various molecules (Chandrasekhar and Bera, 2012). These include ATP (Eltzschig et al., 2006; Kang et al., 2008; Bol et al., 2016), glutamate (Ye et al., 2003), lactate (Karagiannis et al., 2016), NAD^+ (Bruzzone et al., 2001), IP_3 (Gossman and Zhao, 2008), PGE_2 (Cherian et al., 2005; Siller-Jackson et al., 2008; Burra and Jiang, 2009), and glutathione (Rana and Dringen, 2007; Ye et al., 2015) that all potentially may become depleted when the cell is under stress and energy reserves run on empty. All released substances may additionally act as autocrine and paracrine signaling molecules, which has been best documented for ATP (Lohman et al., 2012; Wang et al., 2013b; Lohman and Isakson, 2014). HCs also facilitate molecular entry provided there is a chemical or electrochemical driving force; for example, the fluorescent glucose analog 2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose may enter cells through HCs. Based on data from the Human Metabolome Database, Esseltine and Laird (2016) estimated the number of molecules that may be able to escape through open HCs in the order of several tens of thousands (Esseltine and Laird, 2016), underscoring the potential scope of inappropriate HC opening. The mechanisms that lead to HC opening link to mutational defects that result in constitutively open HCs or to triggers of electrical, chemical, or mechanical nature. Mutations may impact various levels of the connexin life cycle, including connexin transport to the plasma membrane, functions of GJs, or HC functions. Interestingly, some mutations result in a gain-of-function of HCs and a loss-of-function of GJs demonstrating that HCs/GJs, although composed of the same building blocks, can in principle be distinctly modulated. Evidence for such distinct regulation is accumulating and further expanded in this review. Some mutations of Cx43 that result in increased HC function, yielding leaky HCs, are illustrated in Fig. 1 (for review, see Retamal et al., 2015). In addition to constitutive mutational defects, HCs can be triggered to open in response to electrical signals, i.e., changes of the

membrane potential, mechanical forces acting on the plasma membrane (Siller-Jackson et al., 2008; Batra et al., 2014), and chemical signals including the lowering of extracellular Ca^{2+}, increases of intracellular (cytoplasmic) Ca^{2+}, NO, or more complex chemical environments such as proinflammatory conditions or ischemia-reperfusion (reviewed in John et al., 2003; Wang et al., 2013a; Castellano and Eugenin, 2014; Orellana et al., 2014, Schulz et al., 2015; Orellana, 2016).

Pannexins are another protein family composed of three members (Panx1, Panx2, and Panx3) that form channels that resemble connexin hemichannels (Bruzzone et al., 2003). They share the same tertraspan topology, with two ELs, one CL, and N- and C-terminals inside the cell, but lack sequence homology. They are vertebrate analogs of invertebrate innexins that, in contrast to innexins and connexins, generally do not form cell-cell connecting channels as GJs do. As a result, the consensus is to call hexameric channels composed of Panx1, the most common pannexin, just channels and not HCs (Sosinsky et al., 2011). Pannexins have in common with connexin HCs a wide pore, and both act as forefront diffusive ATP release pathways [estimated pore size is 17–21 Å for Panx1, slightly higher than for connexins, and ~30 Å for Panx2, which possibly forms octameric channels (Ambrosi et al., 2010)]. Panx1 has been demonstrated to be important in inflammasome activation (Kanneganti et al., 2007), and Panx1 and Panx2 are reported to be involved in ischemia-induced neuronal cell death [Panx1 (MacVicar and Thompson, 2010; Thompson, 2015; Weilinger et al., 2016); Panx2 (Bargiotas et al., 2011)]. Panx1-triggered ATP release acts as a find-me signal of apoptotic cells, which attracts phagocytic cells to remove dying cells early in the apoptotic process in an orderly manner (Chekeni et al., 2010). In the heart, Panx1 ATP release has been implicated in attracting phagocytes (Oishi et al., 2012) in pathologic fibrosis (Lu et al., 2012a), in activating sympathetic fibers (Dong et al., 2016), in ischemic pre- and postconditioning (Vessey et al., 2011a,b), in atrial fibrillation (Petric et al., 2016), and as a large conductance channel in cultured atrial cardiomyocytes (Kienitz et al., 2011) (roles of cardiac pannexins are reviewed in Li et al., 2015). In blood vessels, Panx1 in smooth muscle cells is involved in the regulation of vascular tone (Billaud et al., 2012), whereas its presence in venous endothelial cells regulates leukocyte migration during inflammation (Lohman et al., 2015; reviewed in Good et al., 2015; Begandt et al., 2017).

Panx1 channels can be opened by caspases-3 and -7, which cleave off part of the CT, and by Src family kinases that target the CT (Chekeni et al., 2010; Thompson, 2015; Weilinger et al., 2016). Most of the connexin channel inhibitors also inhibit pannexin channels (Dahl et al., 2013), but Panx1 is specifically inhibited by ^{10}Panx1, a peptide mimicking a sequence on EL1 of Panx1 (Pelegrin and Surprenant, 2006) and the quinolone antibiotic trovafloxacin, which explains the toxicity and side effects of this drug (Poon et al., 2014). Due to the lack of sequence homology with the connexins, the regulation of Panx1 channels is very different from connexins. For example, Panx1 channel open with truncation of the CT (Chekeni et al., 2010), whereas the CT is essential for Cx43 HC function (De Vuyst et al., 2007; Kang et al., 2008; Ponsaerts et al., 2010) (Fig. 2A). In terms of regulation by posttranslational modifications, pannexins still need to be scrutinized in detail. Most importantly, pannexins function as a plasma membrane channel without necessarily connecting cells, whereas connexins represent the more complicated case because they form junctional channels as well as HCs. For these reasons, in this review we

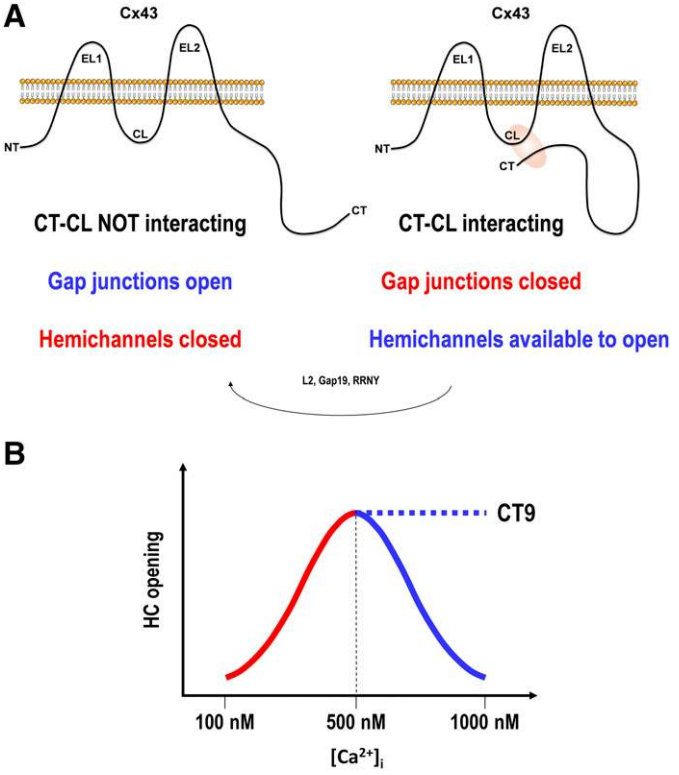

Fig. 2. Functional effects of loop-tail interactions and $[Ca^{2+}]_i$ elevation on Cx43 channel function. (A) Interaction of the connexin C-terminal tail (CT) with the cytoplasmic loop (CL) distinctly influences the function of gap junctions (GJs) and hemichannels (HCs). In the absence of CT-CL interaction, GJs are open while HCs are kept closed. Upon interaction of the CT with the CL, GJs are closed while HCs become available to open. The actual opening of HCs only occurs when a trigger is present, which can be of electrical (changes in membrane potential leading to depolarization or positive voltages) or chemical nature (e.g., changes in extracellular or intracellular Ca^{2+} concentration, inflammatory conditions, ischemic conditions including reperfusion). HC blockers like L2, Gap19 and RRNY (sequences see Fig. 1) bind to the CT and prevent CT-CL interaction, thereby driving HCs from the available to open state to the closed state. At the level of GJs, this prevents the closure of the junctional channels. (B) $[Ca^{2+}]_i$ modulation of Cx43 HC opening. Moderate $[Ca^{2+}]_i$ promotes HC opening via calmodulin-dependent signaling (red part of the bell-shaped curve). High $[Ca^{2+}]_i$ inhibits HC opening by disrupting CT-CL interaction (blue part). CT9 peptide removes the high $[Ca^{2+}]_i$ brake. For details, see *sections II and III*.

chose to concentrate on connexins and limit pannexin discussion to introductory concepts.

This review starts with an introduction on connexins, their expression, life cycle, and regulation (*section II*), followed by a more detailed account on recent insights and substances that modulate connexin channel function (*section III*). We next review the roles of connexins in vascular disease, including atherosclerosis, thrombosis, restenosis, and ischemia-reperfusion injury (*section IV*); in cardiac disease discussing risk factors, ischemia-reperfusion injury, and cardioprotection (*section V*); and atrial and ventricular fibrillation (*section VI*). *Section VII* gives a detailed account on *GJA1* mutations that lead to the clinical syndrome of ODDD. Although a rare disease, it is a resource of mutations that affect Cx43 trafficking as well as channel functions, providing crucial insights into the role of specific Cx43 amino acid positions and domains, in some cases (e.g., I130T) leading to arrhythmogenesis. In the last chapter, we discuss the role of connexins in brain and spinal cord injury, stroke, ocular disease, and neurodegenerative brain disease, which is often associated with vascular alterations (*section VIII*).

Despite the various roles of connexins and their channels in physiology and pathology, the research field is handicapped because all pharmacological agents (inhibitors or promoters) to date have little specificity and often multiple side effects. However, work in the past two decades demonstrates accumulating evidence that peptide-based molecules are the better option in terms of specificity and off-target effects. Peptide studies are the perfect start and opportunity to define a pharmacophore when combined with systematic amino acid substitution approaches and with surface plasmon resonance (or its more recent congener microscale thermophoresis) and NMR studies, collectively allowing for the characterization of crucial peptide-protein interaction domains. Exemplary for such approach are the L2 and AAP peptides that counteract GJ closure, from which several interesting peptidomimetics have been developed (see *section III*). Another example is the CT9 peptide, which promotes the formation of GJs by affecting the Cx43 life cycle, and also affects HC function (see *sections II* and *III*). A second complication in the connexin field is the fact that two types of channels are formed, GJs and HCs, the functions of which are difficult to disentangle, especially in vivo. Recently, several peptides are in the limelight, including L2 and Gap19 peptides that block Cx43 HCs without inhibiting GJs, and Peptide5 that inhibits HCs but not GJs when applied at low concentration (see *section III*). Thus, there is new excitement and expectation in the field that connexin-targeting drugs may at some point join the pharmacological arsenal available for therapeutic applications (Naus and Giaume, 2016).

II. Expression, Life Cycle, and Regulation of Connexins

A. Expression of Connexins

The major, most prominently expressed cardiovascular connexins are Cx37 [*GJA4* gene on chromosome 1 in the human (Söhl and Willecke, 2003)], Cx40 (*GJA5* on chromosome 1), Cx43 (*GJA1* on chromosome 6), and Cx45 (*GJA7* on chromosome 17), with Cx43 being the most abundant isoform. Connexins have been grouped in five families based on their sequence (conserved domains), length of the cytoplasmic loop, and gene structure, each represented by a Greek letter (Cruciani and Mikalsen, 2006; Abascal and Zardoya, 2013). Cx37, Cx40, and Cx43 belong to group α, Cx32 is a β connexin, and Cx45 is a γ connexin. Their regional expression palette differs between the vascular system and the heart. In both, the expression pattern can vary with the species and we mainly focus on the human in what follows.

Blood vessel endothelial cells, including those of the coronary system that perfuses the heart, express Cx37, Cx40, Cx43, and Cx45 but the level or even presence depends on the diameter of the vessels. Cx37 and Cx43 are predominant at all levels up to capillaries, Cx40 is not present in capillary endothelial cells, and Cx45 is only present in large arteries. On the venous side, the connexin expression is low, and another connexin not mentioned yet, Cx32, is found in veins (for reviews, see Haefliger et al., 2000; Begandt et al., 2017). The smooth muscle cells surrounding the endothelium express Cx37, Cx43, and Cx45; Cx40 expression is low or not present.

In heart muscle, five different connexins are represented: Cx31.9, Cx37, Cx40, Cx43, and Cx45. Cx43 is present primarily in working myocytes of the atrium and ventricle (Davis et al., 1994,1995); it is the predominant connexin in ventricular myocardium (Severs et al., 2008). It is also present in the human conduction system but less so in mice and rat (Teunissen and Bierhuizen, 2004). Cx43 also abounds in fibroblasts, which provide the structural skeleton of the myocardium that strongly expands in heart disease (McArthur et al., 2015); GJs may form between cardiomyocytes and fibroblasts, thereby increasing the arrhythmia risk. Cx43 is also found in stem cells used for cardiotherapeutic purposes (Lu et al., 2012b); as such, they may aid in electrically reconnecting cardiomyocytes and promote conduction (Hofshi et al., 2011). Cx40 is present in the atrium and conducting system (except in rats where Cx43 is the main atrial connexin) (Davis et al., 1994; Saffitz et al., 1994; Simon et al., 1998; van Rijen et al., 2001). Cx40 is prominently expressed in the ventricle early in development, but then declines to near absence in the adult (Van Kempen et al., 1996). Levels of Cx40 and Cx43 are very similar in atrial myocytes (Lin et al., 2010). Cx45 is found in many

cardiac regions (Davis et al., 1994), but it may not be abundant; it may be important in defining the developing conduction system (Coppen et al., 1999). Cx31.9 (Cx30.2 in rodents) is a connexin that was discovered relatively recently (Belluardo et al., 2001; Nielsen et al., 2002), which may contribute to atrioventricular nodal impulse conduction (Kreuzberg et al., 2005, 2006) (human Cx31.9 gene is on chromosome 17).

Last, but not least, several types of blood cells also express connexins. Red blood cells do not express connexins but they do express Panx1 (reviewed in Begandt et al., 2017). Platelets express Cx37 and Cx40, with low levels of Cx32 and Cx43 and Panx1 (reviewed in Vaiyapuri et al., 2015; Molica et al., 2017). Cx43 and Cx37 are present in monocytes, and Cx43 is present in neutrophils and T- and B-lymphocytes (reviewed in Pfenniger et al., 2013; Glass et al., 2015).

Vascular alterations are invariably involved in diseases of the central nervous system, including stroke, traumatic injuries, and neurodegenerative disorders. The specific parenchymal connexins in neurons and glial cells are introduced in *section VIII* handling on the role of connexins in cerebrovascular and retinovascular disease.

Transcriptional control of Cx40 and Cx43 genes is known to be mediated by several transcription factors, including Sp1 and Sp3 for Cx40 and Sp1/Sp3 and AP1 for Cx43 (Teunissen and Bierhuizen, 2004). Cx43 transcription is promoted by tetradecanoyl phorbol acetatevia activation of c-jun and c-fos that dimerize to form AP1. Many other signals affect Cx43 expression including thyroid and parathyroid hormones, estrogens (see *section V.B.1*), prostaglandin E2, and signaling via Ras, Wnt1, and cAMP pathways. In tumor cells, connexins like Cx43 and others are often downregulated by hypermethylation of the gene promoters (reviewed in Vinken, 2016). Histone acetylation has the opposite effect and stimulates the expression of Cx43 and Cx45, as induced by chemical inhibitors of histone deacetylase (HDAC) enzymes such as sodium dibutyrate (Hattori et al., 2007). Other HDAC inhibitors like suberoylanilide hydroxaminic acid upregulate Cx37 but downregulate Cx40 in cardiodystrophic mice (Colussi et al., 2010). The nonspecific connexin channel inhibitor 18-α-glycyrrhetinic acid and carbenoxolone have been reported to act via a decreased transcription (Guo et al., 1999; Herrero-González et al., 2009; Wang et al., 2009) (see *section III.A*).

A major point of posttranscriptional control is mediated by microRNAs (miRNAs), which may suppress translation or cleave mRNAs and thereby affect mRNA stability. For the cardiovascular connexin family, most evidence is available for Cx43 and Cx40. Cx43 targeting miRNAs bind to the 3′-UTR region in the mRNA (reviewed in Vinken, 2016) and a prominent example is miR-1. When overexpressed in rat heart, miR-1 exacerbates arrhythmogenesis by reducing Cx43 expression as well as expression of Kir2.1 K^+ channel subunits (Yang et al., 2007). Interestingly, miR-1 is overexpressed in individuals with coronary artery disease, whereas it is downregulated in patients with tetralogy of Fallot (Wu et al., 2014). Other miRNAs that downregulate Cx43 and lead to arrhythmogenesis are miR-17-92 and miR-130a (Danielson et al., 2013; Osbourne et al., 2014) (see also *section V*). Some miRNAs promote connexin expression, such as miR-208a that stimulates cardiac Cx40 expression (Callis et al., 2009). Adenylate/uridine-rich elements and RNA-binding proteins also affect mRNA stability, but they have been less characterized in a cardiovascular context (reviewed in Salat-Canela et al., 2015).

B. The Life Cycle from Connexins to Channels and Back

The connexin life cycle encompasses various steps, including 1) connexin trafficking and formation of HCs, 2) GJ assembly, 3) formation of GJ plaques, and 4) disassembly of GJs by internalization and degradation. These various steps are introduced below; their modulation is discussed further in *section II.F* (for reviews, see Laird, 2006; Salameh, 2006; Thevenin et al., 2013).

1. Connexin Trafficking and Formation of Hemichannels. After biosynthesis in the ER, connexins are transported by forward trafficking to the plasma membrane via the secretory pathway involving the Golgi apparatus. Most connexins follow this pathway but trafficking for Cx26 (a connexin found in liver, cochlea, Schwann cells, and oligodendrocytes) has been reported to be Golgi dependent (Thomas et al., 2005) as well as independent (Martin et al., 2001). Hemichannels are hexameric connexin assemblies that are formed by oligomerization that starts in the endoplasmic reticulum, continues in the Golgi apparatus, and stabilizes in the trans-Golgi network (Laird, 2006). Some connexins like Cx43, the predominant cardiovascular connexin, and Cx46 (an eye lens connexin) appear monomeric in the ER/Golgi and only hexamerize in the trans-Golgi network (Musil and Goodenough, 1993; Koval et al., 1997). In Cx26, the smallest and best characterized connexin in terms of atomic structure (3.5 Å resolution based on X-ray crystallographic analysis), the subunit interactions that lead to oligomerization involve the outer half of transmembrane helices TM2 (a pore lining domain) and TM4 (a membrane facing domain) as well as the ELs (Maeda et al., 2009). Additionally, amino acids in the CL, close to the transition into TM3 are also involved (Smith et al., 2012). Arg-184 in Cx26 (EL2) is a crucial amino acid that, when mutated, leads to disturbed oligomerization as do mutants of Arg-75 (EL1, just before transitioning into TM2) (Maeda and Tsukihara, 2011); both Arg are well conserved in other connexins.

Vesicles containing HCs, at least those composed of Cx43, are transported along microtubules and actin

filaments to the plasma membrane, and tethering of the microtubule plus ends at the adherens junction proteins promotes the plasma membrane delivery process (Shaw et al., 2007). Connexins undergo various posttranslational modifications, including extensive regulation via phosphorylation (see *section II.F*). Two kinases are involved in connexin trafficking, PKA and PKB (Akt kinase). Increased cyclic AMP and subsequent PKA activation with Cx43 phosphorylation at Ser-364 and Ser-365 promotes trafficking and hence GJ formation (Paulson et al., 2000; Shah et al., 2002); PKB/Akt phosphorylation at Ser-373 promotes trafficking through involvement of 14-3-3 proteins (Park et al., 2007) (a more detailed discussion is given in *section II.F*).

Once in the plasma membrane, HCs are kept in a closed state by two mechanisms that relate to the membrane potential and the Ca^{2+} concentration inside and outside the cell. HCs are typically closed at negative membrane potential (V_m) as well as by the 1–2 mM extracellular Ca^{2+} concentration ($[Ca^{2+}]_e$) (Trexler et al., 1996; Contreras et al., 2003; Verselis et al., 2009). Also in the ER, the millimolar $[Ca^{2+}]$ inside keeps the HCs closed to avoid ER Ca^{2+} store leakage. Interestingly, GJ formation discussed next is also dependent on $[Ca^{2+}]_e$ and involves E-cadherin (Jongen et al., 1991).

2. Gap Junction Channel Assembly. The next step in the connexin lifetime consists of the head-to-head docking of two HCs from adjacent cells to assemble as a GJ channel. It is currently not known whether and to what extent a separate HC pool exists that is not destined to become incorporated into GJs. Hemichannels on their way to GJs have been proposed to move on lipid rafts until they meet another HC to dock with Schubert et al. (2002). HC docking involves complex interactions between the ELs of the apposed proteins, resulting in the formation of a sealed conduit between the HC heads, located in the 40-Å-wide gap area that separates the two plasma membranes. At this occasion, the two interacting HCs open by the opening of a loop gate (Bukauskas et al., 1995; further discussed under *section II.E*). Based on 3.5 Å resolution X-ray crystallographic data from Cx26 and work from other authors referred to below, the molecular details of EL interactions can be summarized as follows. First, the ELs within a HC form an anti-parallel β sheet stabilized by three disulfide bridges between Cys residues conserved in all connexins, which connect EL1 and EL2 from each single subunit by intramolecular interactions, thereby maintaining a rigid tertiary structure that favors docking (see Fig. 9 in Foote et al., 1998; reviewed in Sosinsky and Nicholson, 2005; Yeager and Harris, 2007; Maeda and Tsukihara, 2011). Second, based on the Cx26 crystal structure, the interaction between the ELs of opposed HCs is thought to occur such that the EL protrusions from an HC fit into the valleys between ELs of an HC at the opposite side, i.e., each connexin subunit can interact with two subunits of the opposed connexon, forming a β barrel that seals the inside of the channel from the extracellular space (Foote et al., 1998; Yeager, 1998; Yeager and Harris, 2007; Maeda et al., 2009). The inner wall of this junctional structure is formed by the interaction of opposed EL1 domains of each connexin subunit, whereas the outer wall is formed by interacting EL2 domains of each connexin (illustrated in Fig. 1 in Riquelme et al., 2013). Based on a rotation of 30° between the ELs of opposed HCs, an alternative scenario whereby EL1 interacts with an opposed EL2, and EL2 with an opposed EL1, has also been suggested (illustrated in Fig. 4 in Perkins et al., 1998). The EL sequences are highly conserved between different connexins, and data from Cx26 indicate that intercellular EL1-EL1 interactions involve hydrogen bond formation between a highly conserved Asn-54 at one side with Leu-56 at the opposite side, as well as between opposed Gln-57 residues. EL2-EL2 interactions involve hydrogen bonds and salt bridges formed between Lys-168, Asp-179, and Thr-177 from one side and with Asn-176 from the opposite side (Maeda et al., 2009) (Fig. 1).

3. Formation of Gap Junction Plaques. GJ channels organize in so called plaques that have a size that ranges from just visible by confocal microscopy up to several micrometers in length and contain hundreds to thousands of GJ channels (Klaunig and Shi, 2009) separated by a center-to-center distance of ~10 nm (Goodenough and Revel, 1970; McNutt and Weinstein, 1970). Very little is known on how exactly GJ channels interact with each other to be arranged in a densely packed well-organized array of a GJ plaque (atomic force microscopy images of gap junction plaques can be found in Müller et al., 2002; Yu et al., 2007). An ill-defined minimum number of channels or channels per plaque is necessary to obtain electrical or dye coupling (Palacios-Prado et al., 2009). Surprisingly, only a limited number of channels in the order of 1% are proposed to be open in a plaque [Cx36 (Marandykina et al., 2013); Cx57 (Palacios-Prado et al., 2009)]. The inclusion of newly assembled GJs in a plaque is an organized event that involves interactions with the zonula occludens 1 (ZO-1) scaffolding protein, which links Cx43 to F-actin. ZO-1 has three PDZ domains and Cx43, as most other connexins, interacts with the PDZ2 (see Thevenin et al., 2013 for more details). Exceptions are Cx36 (a connexin found in neurons and the endocrine pancreas), which interacts with the PDZ1 domain (Li et al., 2004c), Cx45 (a heart and skin connexin) that interacts with all three PDZ domains (Kausalya et al., 2001), and Cx32 (a liver connexin also found in Schwann cells), which interacts with ZO-1 domains other than PDZ or indirectly via intermediate partners (Kojima et al., 2001; Li et al., 2004b). Cx43 interacts with PDZ2 via a DLEI sequence located at the C-terminal end (see Fig. 1), with the last four amino acids being crucial; removal of the last amino acid (Ile-382) prevents the

interaction (Jin et al., 2004). Cx43 HCs are linked to ZO-1 proteins at the periphery of a GJ plaque in a zone called the perinexus that is distinct from the centrally located "nexus" structure that contains the GJs (Rhett et al., 2011; Rhett and Gourdie, 2012). The dynamics of plaque formation is incompletely understood; it involves accretion of newly formed GJs at the outer edge of the plaque, whereas old GJs are removed from the center (Gaietta et al., 2002; Lauf et al., 2002), probably by endocytosis of small, or even large, circular double membrane vesicles called "annular junctions" or "connexosomes" (Archard and Denys, 1979; Jordan et al., 2001; Falk et al., 2009). Outer rim accretion is a two-step process whereby HCs assemble as GJs and GJs incorporate into plaques; the connexin−ZO-1 interaction is crucial in this process. When Cx43 is tagged with GFP at its CT end, a domain involved in interaction with ZO-1, GJ plaques grow significantly larger (Hunter et al., 2003). Additionally, adding a peptide composed of the last nine amino acids of the Cx43 CT (Arg-374–Ile-382 further called CT9, see Fig. 1) and linked to an antennapedia internalization sequence to make it membrane permeable (called αCT1) has the same plaque increasing effect (Hunter et al., 2005). This and other work from the Gourdie group, making use of various approaches, including duolink (proximity ligation) protein-protein interaction studies, demonstrates that HCs are linked to ZO-1 and that disruption of this linkage is crucial in the process of HC incorporation and GJ assembly into the plaque (reviewed in Palatinus et al., 2012; Rhett et al., 2013). Given the multitude of scaffolding and cytoskeletal links to the CT, other proteins and structures such as microtubules (Toyofuku et al., 1998; Giepmans et al., 2001) and myosin VI (Waxse et al., 2017) may also be involved. At least two kinases are involved in GJ assembly and incorporation in GJ plaques: PKB/Akt and casein kinase (CK)1. PKB/Akt phosphorylation of Ser-372 or -373 helps in loosening the binding of Cx43 to ZO-1 (Chen et al., 2008); CK1 phosphorylation of Ser-325, Ser-328, and Ser-330 promotes GJ/plaque assembly (Cooper and Lampe, 2002) and promotes dye coupling and GJ channel opening (Lampe et al., 2006). Of note, ZO-1 interactions with the Cx43 CT are not necessary for HC/GJ formation (Fishman et al., 1991; Dunham et al., 1992). ZO-1–CT interactions are modulated by various influences: 1) c-Src interacts with the Src homology domain 3 (SH3) domain on the Cx43 CT [Pro-274–Pro-284 (Kanemitsu et al., 1997), see Fig. 1], and this displaces the ZO-1–CT interaction that is located ~100 amino acids away from the SH3 domain (Sorgen et al., 2004); 2) ZO-1–CT interactions decrease upon acidification, and this favors c-Src binding to CT (Duffy et al., 2004); and 3) ZO-1–CT interactions increase upon isolating cardiomyocytes from their native environment and are thus involved in remodeling (Barker et al., 2002).

4. Gap Junction Disassembly: Internalization and Degradation. Several possible scenarios exist for GJ removal. Early work suggested dispersal of junctional particles in the plasma membrane, away from the junction (Lane and Swales, 1980). It has also been proposed that GJs may split into reseparated HCs according to an unzippering scenario. GJ unzippering has been suggested as a mechanism for peptide inhibitors of GJ channels like Gap26 or Gap27 (see *section III*) (Berthoud et al., 2000), but there is currently no hard evidence to support such a mechanism. Possibly, HCs are also internalized: making use of a fluorescently labeled peptide identical to a Cx43 EL1 sequence and CFP-tagged Cx43, Dermietzel et al. (2003) demonstrated uptake of double-labeled structures, indicating inward trafficking of HCs (Dermietzel et al., 2003). However, the currently best characterized uptake scenario consists of the uptake of an entire GJ plaque (or parts of it), which then appears in one of the cells as an annular junction (Jordan et al., 2001; Piehl et al., 2007; Falk et al., 2009). The uptake process can occur by clathrin (Gumpert et al., 2008)- or caveolin-dependent (Schubert et al., 2002; Lin et al., 2003b) endocytosis (reviewed in Thevenin et al., 2013). Clathrin-dependent uptake involves several clathrin adapter proteins such as adaptor protein complex-2 that interact with Tyr-based sorting signals located on the CT tail (in particular Y^{265}AYF, Y^{286}KLV, and others for Cx43; see Fig. 1) and disabled2. Dynamin2, a GTPase, facilitates the inward pinching-off of plasma membrane vesicles, whereas myosin-VI provides vectorial inwardly directed transport. Further breakdown can occur via lysosomal or proteasomal pathways (Laing and Beyer, 1995; Laing et al., 1997,1998; Qin et al., 2003, reviewed in Salameh, 2006). Ubiquitination is central for proteasomal breakdown, but proteasomal breakdown of Cx43 can occur in the absence of ubiquitination (Su et al., 2010; Su and Lau, 2012); ubiquitination can furthermore be involved in endosomal/lysosomal breakdown. Ubiquitination occurs at Lys residues, of which Cx43 has 23; 20 of them are located on the CL and CT, which contain the more likely ubiquitination sites. The large GJ structures are not typical targets for proteasomal breakdown, but Girão et al. (2009) reported that a Cx43-ubiquitin fusion protein present in HCs and GJ plaques was internalized by a process that involves the ubiquitin ligase Nedd4 and the endocytic adapter protein Eps15. Nedd4 interaction with Cx43 occurred at a Pro-rich motif in the CT that overlaps with the Tyr-286 based sorting signal (Y^{286}KLV) (Fig. 1) (Leykauf et al., 2006; Girão et al., 2009). Mutation of Tyr-286 and of downstream Val-289 increases the Cx43 half-life and stability of the GJs (Thomas et al., 2003; Catarino et al., 2011). Ubiquitination may also play a role in the endosomal/lysosomal pathway where it may target endosomes for lysosomal breakdown via the Tsg101 sorting protein that also binds to Cx43 (Auth et al., 2009; Leithe et al., 2009). In

addition to proteasomal and lysosomal degradation, growing evidence indicates involvement of autophagic degradation of connexins as well (Lichtenstein et al., 2011; Bejarano et al., 2012; Falk et al., 2012; Fong et al., 2012; Iyyathurai et al., 2016). These various removal/internalization pathways offer interesting, yet unexplored, handles to modulate GJ size (Beyer and Berthoud, 2002).

C. Non-Channel Functions of Connexins

Connexins are best known for their function as channels, GJs and HCs. However, connexin proteins are also endowed with channel-independent functions. Many, but not all, of the below described non-channel effects link to the CT tail of Cx43, which is a hub for interactions with other scaffolding or signaling proteins (Giepmans, 2004; Hervé et al., 2012) and also the site by preference for modulatory phosphorylation events. The list of non-channel functions includes connexin interactions with cytoplasmic signaling molecules, connexin-linked sequestering of transcription factors, CT-migration to the nucleus, and adhesion aspects linked to the mechanical cell-cell connection provided by GJs.

In terms of interaction with cytoplasmic signaling molecules, there is evidence that connexins interact with apoptotic factors thereby influencing cell death. Cx26 and Cx43 colocalize with the Bcl-2 proteins Bak, Bcl-xL, and Bax in the cytoplasm of human breast and colorectal cancer cells (Kanczuga-Koda et al., 2005a,b). Recent evidence demonstrates a more direct interaction of Cx43 with apoptosis signal-regulating kinase 1 (Giardina et al., 2007). Another example comes from the glioma brain tumor field. In these tumors, the levels of Cx43 decrease with increasing malignancy (Sin et al., 2012). The inverse relation between tumor cell proliferation and Cx43 expression in part links to the fact that Cx43, in particular the CT tail, exerts a brake on cell proliferation (Zhang et al., 2003). Work from the Tabernero group recently demonstrated that Cx43 inhibits proliferation by interacting with the proto-oncogene Tyr protein kinase c-Src via a CT-located SH3 domain (see Fig. 1), which involves corecruitment of the phosphatase and tensin homolog and CT Src kinase (González-Sánchez et al., 2016; reviewed in (Tabernero et al., 2016). Interestingly, a peptide composed of Ala-266 to Pro-283 [PEP-2 (Gangoso et al., 2014); see Fig. 1] was able to mimic the effect of the full Cx43 protein in limiting the proliferation glioma stem cells (González-Sánchez et al., 2016); more recent work demonstrated that PEP-2 was able to reduce migration, invasion, and survival of primary glioma stem cells isolated from human glioma tumor samples (Jaraiz-Rodriguez et al., 2017).

In terms of signaling to the nucleus, there are two possibilities: either the CT is involved in sequestering cytoplasmic transcription factors in a direct/indirect manner, or the CT itself translocates to the nucleus. The transcription factor ZONAB (ZO-1-associated nucleic acid binding protein) is an example of the first possibility. ZONAB associates, through its ZO-1 binding partner, with the glial connexins Cx30, Cx43, and Cx47 (Penes et al., 2005; Li et al., 2008b). ZONAB not linked to ZO-1 migrates to the nucleus where it acts as a transcription factor that binds to promoter sequences containing an inverted CCAAT box (Balda and Matter, 2000) and modulates the cell cycle, mostly resulting in increased proliferation (reviewed in Zahraoui, 2004). The ZONAB link to connexins and cell proliferation is thus indirect and based on evidence from the tight junction field; in any case ZONAB-sequestering by Cx30, Cx43 and Cx47 may be involved in inhibition of cell proliferation by these connexins. Next to ZO-1 sequestering of ZONAB, Cx43 also interacts with ZO-2 (Singh et al., 2005), which sequesters the transcription factors c-jun, c-fos and CCAAT enhancer binding protein (Betanzos et al., 2004), again resulting in decreased cell cycle activity and proliferation (Tapia et al., 2009). Another example of connexin-sequestering comes from the cell-cell adhesion molecule β-catenin. Cx43 interacts with β-catenin (Ai et al., 2000) and in this way influences Wnt/β-catenin signaling, which acts to promote cell cycling and proliferation. Thus, Cx43–β-catenin interaction reduces β-catenin availability, inhibiting its migration to the nucleus and its subsequent interaction with transcription factors, thereby decreasing cell proliferation (MacDonald et al., 2009). Direct migration of the Cx43 CT to the nucleus has also been implicated in the Cx43-suppressive effect on cell proliferation despite the fact that the CT does not contain a known nuclear target sequence (Dang et al., 2003).

Connexins are also endowed with cell adhesion functions (Lin et al., 2002; reviewed in Prochnow and Dermietzel, 2008). The Kriegstein group demonstrated that the radial migration of excitatory neuronal precursor cells along radial glial cells during cortical development depended on adhesive properties provided by GJs composed of Cx26 and Cx43 (Elias et al., 2007). Follow up work showed this was also the case for the more complex migration pathway of inhibitory neuronal precursors, which come from lateral and make a switch in radial direction upon interacting with radial glia (Elias et al., 2010; reviewed in Elias and Kriegstein, 2008). Work from the Naus group demonstrated that adhesion-linked connexin properties also play a role in the migration and invasion of glioma cells (Sin et al., 2016; reviewed in Matsuuchi and Naus, 2013; Naus et al., 2016). Cx43-mediated adhesion effects have also been reported for the spreading of B-lymphocyte adhesion when cultured on a substrate (Machtaler et al., 2011). Forced expression of Cx43 in prostate cancer cells was furthermore found to promote metastasis to the bone, which strongly expresses Cx43 (Lamiche et al.,

2012). This suggests that the docking of two apposed HCs and/or the function of GJs may play a role in tumor metastasis to specific organs. Breast tumor carcinoma cells express Cx43 and frequently metastasize to the brain; recent work has provided evidence that breast metastatic carcinoma cells take advantage of GJ communication with Cx43-expressing astrocytes, demonstrating that the role of connexins in metastasis definitely involves channel functions (Chen et al., 2016).

Last but not least, alterations in all aspects of the connexin life cycle (see *section II.B*) may in some way be linked to non-channel effects. For example, changes in connexin expression or the presence of mutant connexins may affect the trafficking of other proteins that follow the secretory pathway and thereby influence intracellular vesicle trafficking and the release of exosomes, which also contain Cx43 (Soares et al., 2015). A striking example comes from the heart, where the loss of Cx43 expression leads to reduced Na^+ currents in ventricular (Danik et al., 2008; Jansen et al., 2012) and atrial cardiomyocytes (Desplantez et al., 2012a). Similar findings of a reduced Na^+ current, further complicated by ventricular fibrillation, were observed in a cardiac knock-in model of Cx43 that lacked the last five amino acids in the CT [Cx43D378stop mutation (Lübkemeier et al., 2013)]. Work of the Delmar group in this model demonstrated that, although GJ coupling is normal, $Na_V1.5$ delivery to the plasma membrane was impaired in a microtubule-dependent manner (Agullo-Pascual et al., 2014b). Omission of the five last Cx43 CT amino acids was found to limit the capture of the microtubule plus end tracking protein end-binding-1 at the ID, resulting in impaired cargo delivery to this location (reviewed in Leo-Macias et al., 2016).

Further reviews on non-channel functions of connexins can be found in Giepmans (2004), Jiang and Gu (2005), Dbouk et al. (2009); Hervé et al. (2012), Vinken et al. (2012), Zhou and Jiang (2014); Table 1 of Zhou and Jiang (2014) gives an overview of channel-independent effects.

D. Permeability

The structure of HCs and GJ channels composed of Cx26 has been studied in much detail, making use of three-dimensional X-ray crystallographic analysis at 3.5 Å resolution (Maeda et al., 2009; reviewed in Maeda and Tsukihara, 2011). These studies suggested that the smallest pore diameter of the Cx26 channel is ~14 Å. As a consequence, Na^+, K^+, and Cl^- can pass through in the presence of their hydration shell [diameters 3.58, 3.31, and 3.32 Å, respectively (Volkov et al., 1997)]. The low-pass cut-off for a 14-Å-wide pore is in the order of ~1.2 kDa MM based on an empirically derived relation between diameter and molecular weight for globular proteins (Erickson, 2009). Based on size exclusion studies with Alexa dyes, the pore diameter of Cx43 channels was estimated to be in the order of 14.8 Å (Weber et al., 2004), giving a calculated ~1.4 kDa MM cut-off. For Cx37, size cut-off based on polyethylene glycols was in the 6.8–8 Å range (Gong and Nicholson, 2001). In addition to size, permeation also depends on charge. Although the narrowest diameter of the pore is much wider than hydrated Na^+ or Cl^-, the permeability for these ions can be different, indicating charge selectivity, even for atomic ions (Veenstra et al., 1995). For larger charged molecules, which are more likely to interact with the channel wall, charge selectivity is even more pronounced and sometimes very different between different connexins. For example, negatively charged molecules like carboxyfluorescein (8.2 Å, charge −2) or Lucifer yellow (9.9 Å, charge −2) permeate better through GJ channels composed of Cx43 than those composed of Cx40. By contrast, positively charged molecules like ethidium bromide (10.3 Å, +1) permeate better through Cx26-based channels (Kanaporis et al., 2011). Despite the importance of size and charge, there are no obvious rules to explain the permeation of metabolic or signaling molecules, suggesting that still unknown interactions (electrostatic, van der Waals, or covalent in nature) occur within the channel pore (Weber et al., 2004; reviewed in Harris, 2007). As a result, channel permeation does not occur along a free diffusion scenario but based on selectivity properties that are still poorly understood (Ek-Vitorin and Burt, 2013). For example, Cx26 channels are more or less equally permeable to cAMP (MM 329) and IP_3 (MM 420) despite the large difference in charge (neutral and −6, respectively) (Hernandez et al., 2007). ATP (MM 507, charge −4) permeates a 300-fold better through Cx43 channels than through Cx32 channels (Goldberg et al., 2002), a huge difference probably difficult to explain on pore size only. For cAMP, the permeation sequence is Cx43 > Cx26 > Cx45 = Cx32 (Wang and Veenstra, 1997; Bedner et al., 2003, 2006). Additionally, the permeability for fluorescent dye molecules does not always go hand in hand with the channel electrical conductance properties (fully open state versus substrates; see *section II.E*) (Brink et al., 2006; Eckert, 2006) and high conductance channels like those formed by Cx37 display poor permeation of fluorescent dyes (Veenstra et al., 1994, 1995). Clearly, connexin channels display connexin-specific permeability profiles, and more studies are needed to have a better understanding of both the exact pore size (based on structural and functional approaches) and the nature of the interactions of the permeant with the channel. This is especially relevant for the larger permeating substances that are more likely to interact with the channel pore-lining TM1 and TM2 domains compared with atomic ions that have more space available and therefore are able to diffuse more freely with less interactions with the channel wall-lining residues; when the diameter of the permeant comes in the range of the pore diameter, interactions

with the channel wall will rather determine the permeability profile.

Connexin channels can also be formed by different connexins, resulting in heteromeric or heterotypic channels. HCs can be homomeric or heteromeric (composed of different connexins). Two homomeric HCs make a homotypic GJ channel. A heterotypic GJ channel can be formed by two HCs each composed of a different connexin or by two heteromeric HCs. Two heteromeric HCs may also form a homotypic GJ channel when each subunit pairs with a subunit composed of the same connexin isoform (reviewed in Koval et al., 2014; the various configurations are illustrated in Fig. 1 of Mese et al., 2007). The most studied heteromeric/heterotypic GJs are formed by the cardiovascular connexins Cx37, Cx40, Cx43, and Cx45, which can form heteromeric (He et al., 1999; Beyer et al., 2013) and heterotypic GJs (Lin et al., 2014). The compatibility of different connexins to form heteromeric/heterotypic channels is determined by motifs located at the CL-TM3 transition and left half of EL2. Importantly, heteromericity largely influences GJ permeability for metabolites or signaling molecules, thereby allowing the fine-tuning of the repertoire of substances exchanged between cells (Harris, 2007). Interestingly, heterotypic channels display electrical rectification behavior that may facilitate transport and electrical conduction in a preferential direction (Rackauskas et al., 2007; reviewed in Harris, 2002).

Connexin channels are often called aqueous pores, meaning they allow ions in aqueous solution to pass through the pore surrounded by their hydration shell, as mentioned earlier. In principle, water is able to pass through the channel but only little information is currently available demonstrating direct water flow through the channels. In the lens, GJs composed of Cx46 and Cx50 have been reported to be involved in water flow (Gao et al., 2011). Work on HCs seemed to confirm this, based on cell swelling observed in cell expression systems [Cx43 (Quist et al., 2000); Cx46 (Paul et al., 1991)]. However, more detailed analysis performed on HCs composed of Cx30 or Cx43 has demonstrated they are not water permeable (Hansen et al., 2014). Taken together, connexin channels may be involved in water fluxes, but this is probably the result of ionic flow through GJs or HCs with subsequent osmotic water flow through other pathways, e.g., via aquaporins.

E. Gating

Connexin channels are modulated by numerous influences that link to the cell state or internal/external influences, which most commonly link to voltage, pH, Ca^{2+} concentration, phosphorylation state, and redox state. Several conditions, especially inflammatory conditions, strongly affect connexin channels. Connexin channel permeability expresses the degree of permeation of noncharged permeants, whereas conductance expresses the degree of permeation of charged permeants, i.e., the ease of current passage. Macroscopic conductance is the conductance of an array of channels; modulatory influences alter the macroscopic conductance, which can be caused by changes in the number of channels present, the conductance of single channels, or their gating, i.e., opening and closing activities. Single channel conductance (γ) and gating properties are determined in voltage-clamp experiments, dual cell voltage-clamp for GJ channels and single cell patch-clamp experiments for HCs. These experiments further bring up important information on other biophysical properties like the rise time for opening/closing transitions and the reversal potential, i.e., the potential at which the current reverses direction. Because connexin channels have no selectivity for atomic ions like Na^+, K^+, Ca^{2+}, and Cl^-, the reversal potential is in most cases ~0 mV. The single channel conductance is a fundamental property that differs among the different members of the connexin protein family; hence it can be used to determine what connexin is recorded from (an overview of single channel conductances of HCs and GJs is given in Table 3 of Sáez et al., 2005). Below follows a brief overview on the gating of GJs and HCs by voltage, Ca^{2+}, and pH. Connexin channel gating is a very complex field, in part because the gating, as well as other biophysical properties, differ substantially between different connexins. For that reason, observations in channels composed of Cx26, Cx32, Cx45, or Cx46 to name some, are not necessarily true for Cx43, which we keep as the prototypic example case for this review. Excellent entries into the connexin gating subject can be found in Bukauskas and Verselis (2004), Sáez et al. (2005), González et al. (2007), Moreno and Lau (2007), Ek-Vitorin and Burt (2013), Fasciani et al. (2013), Oshima (2014), and Oh and Bargiello (2015). What follows below is an overview of the most salient aspects of gating, highlighting some proposed structural correlates.

1. Slow Loop Gating and Fast Gating. HCs are normally closed but open upon docking with an apposed HC by a process of loop gating (Bukauskas et al., 1995; Trexler et al., 1996; Contreras et al., 2003), which involves movements of residues at the border of TM1/EL1 [in particular at position 43–50 for Cx50 (Verselis et al., 2009)] and is characterized by slow gating (transition time ≥10 ms) (reviewed in Bukauskas and Verselis, 2004). Loop gating involves large conformational changes, in particular in the first half of EL1 (Bargiello et al., 2012). Loop gating is associated with various aspects, including 1) the docking process of two HCs per se, 2) the change in voltage sensed by the two HCs upon assembling into a GJ, and 3) the change in $[Ca^{2+}]$ at the external side of the HC upon GJ assembly. The first aspect links to the

interaction of the ELs during docking, which can be seen as ligand-induced gating (Trexler et al., 1996). The second aspect links to the fact that the negative V_m sensed by the HCs, which keeps them closed, disappears upon formation of a GJ channel between two cells with equal (or approximately equal) V_m. The third aspect links to the lowering of $[Ca^{2+}]$ sensed by the outer half of the HC upon docking and sealing-off the channel interior from the extracellular space where $[Ca^{2+}]$ is in the millimolar range (see more in *section II.E.4*). GJs start to close (with slow kinetics taking hundreds of milliseconds to seconds) when the junctional potential difference (V_j), i.e., the difference in V_m between the cells connected by the GJ, starts deviating from 0 mV (reviewed in Bukauskas and Verselis, 2004; Palacios-Prado and Bukauskas, 2012). As a result, the relation between V_j (abscissa) and GJ macroscopic current (ordinate) is a convex-up bell-shaped curve centered around the ordinate, with channel closing at strongly negative or positive voltages (see Fig. 2 in Bukauskas and Verselis, 2004). Single channel analysis has demonstrated that V_j closure of GJ channels is, in contrast to slow loop gating, mediated by fast gating (transition time <2 ms) by a gate that is different from the loop gate (Bukauskas and Verselis, 2004). Slow gating is also involved in the closing process, in particular at the top of the bell-shaped GJ voltage dependence curve (around V_j = 0 mV, where gating starts closing fully open GJ channels) as well as at edges where full closure of the channels is at stake (see Fig. 2 in Bukauskas and Verselis, 2004). As alluded to above, HCs not incorporated into GJs can be opened by depolarizing or positive membrane potentials (Sáez et al., 2005); HC gating also displays slow and fast transitions just like GJs (Contreras et al., 2003). The majority of the gating state transitions of GJs/HCs is slow and only transitions to a residual (substrate) conductance, characterized by a γ that is lower than the fully open channel (~60 pS for Cx43 HCs), are fast. The slow gate always opens with depolarizing or positive voltages (V_m); the fast gate opens with positive V_m for Cx32 but closes with positive V_m for Cx26. As a result, Cx32 HCs increase their open probability with increasing positive voltages; in contrast, Cx26 HCs open with positive voltages but start to close again at increasing positivity, displaying a typical bipolar voltage-dependence (González et al., 2007; Fasciani et al., 2013). Cx43 HCs reportedly also display a bipolar voltage-dependence; however, although slow gate opening clearly increases with above threshold positive membrane potentials, the behavior of the fast gate is more difficult to study because of the paucity of closing events to the residual state.

At the electrical level, GJs behave, at least in a first approximation, as a linear assembly of two apposed HCs; their conduction is half the conduction of the corresponding HC and the gates appear to retain their fast and slow gating polarities in GJs as in HCs (González et al., 2007). A factor that complicates things is the fact that the electrical field sensed by each of the two HCs in a GJ depends on the closed/open state of these HCs (Paulauskas et al., 2012). Of note, GJs do not always behave as a superposition of two HCs, and the responses of both channel types to chemical signals can sometimes be opposite, as discussed below in the context of $[Ca^{2+}]_i$ influences on channel gating. The subconductance state is considered the ground state for electrical gating mediated by fast gating (Valiunas et al., 1997; Ek-Vitorin and Burt, 2013). The ground state for "chemical gating" is the fully closed state and transitions to the fully open state are characterized by slow gating (Ek-Vitorin and Burt, 2013). Two of the most studied examples of chemical gating concern the influence of pH and Ca^{2+} on GJs/HCs, discussed below.

2. Intracellular pH Effects on Gating: The Role of C-Terminal Tail-Cytoplasmic Loop Interaction. GJs close with intracellular acidification (pH 5–6), as occurs in the context of ischemia. Interestingly, when the CT of Cx43 is truncated at Ala-257 (last 125 amino acids, including Ala-257), GJ closure upon acidification disappears (Liu et al., 1993). Moreover, re-expression of the truncated CT part rescues pH_i sensitivity, indicating involvement of the truncated CT part in channel closure (Morley et al., 1996). Based on these observations, a model of Cx43 CT-CL interaction was proposed whereby the CT binds to a receptor structure, the L2 domain located in the second (CT directed) half of the CL (Asp-119 to Lys-144; Fig. 1). This intramolecular interaction was proposed to close the channel according to a ball-and-chain scenario, analogous to the ball-and-chain mechanism for the closure of Na^+ and K^+ channels. The binding of the CT to the L2 domain is facilitated under low pH conditions, as a result of a higher α-helical order in the L2 domain (Duffy et al., 2002); thus, CT-CL interaction and its consequences are strengthened by acidosis. Interestingly, CT truncation at residue 257 removed the residual state of the channel, suggesting that the ball-and-chain CT-CL interaction may act as a fast gate (Moreno et al., 2002). Even more interesting were the observations that addition of the L2 domain as an exogenous peptide decreased the transitions from the fully open state to the residual state and increased the open time of the GJ channels (Seki et al., 2004). Mutation of His-142, located in the L2 domain, to Glu-142 has a similar effect (Shibayama et al., 2006a). Thus, intramolecular CT-CL interactions close GJs upon acidification and addition of the L2 peptide prevents this closure. Substantial data are available in terms of the location of the CT domains that interact with the L2 domain. Morley et al. (1997) reported (based on deletion studies) that the regions Cys-260–Asn-300 and Arg-374–Ile-382 from the CT domain were crucial (Morley et al., 1997) (Arg-374–Ile-382 corresponds to CT9, see Fig. 1). Follow up work demonstrated that a peptide composed of Cys-271–Lys-287

(called C271-K287 peptide in Fig. 1) could prevent acidification-induced uncoupling (Calero et al., 1998). Duffy et al. (2002) showed that three CT peptides with sequences Cys-271–Lys-287, Asp-336–Gly-350, and Lys-346–Asp-360 (mouse sequences) all interacted with L2 in surface plasmon resonance experiments (Duffy et al., 2002). The first two of these peptides, respectively, cover the SH3 domain (Pro-274–Pro-284) and an α-helical domain (Asp-340–Ala-348); the third one is situated between the α-helical domain and the CT9 sequence (see Fig. 1 for the location of these domains). Of note, the Cx43 CT contains two α-helical domains, Ala-315–Thr-326 and Asp-340–Ala-348 (illustrated as two yellow colored domains in Fig. 1), with the rest of the CT being an intrinsically disordered structure (Sorgen et al., 2004). Combined surface plasmon resonance and NMR evidence has indicated that CL interaction with the CT involves the second α-helical domain and a region of the last 19 amino acids of the CT that includes the CT9 domain (Hirst-Jensen et al., 2007). In line with this, RXP-E peptide, discovered through a phage display search for high affinity Cx43 CT binders, was reported to interact with residues 343–346 (within the second α-helical CT domain) and 376–379 (within the CT9 domain; see Fig. 1); this peptide partially prevented acidification- and octanol-induced GJ closure (Shibayama et al., 2006b).

Evidence for involvement of CT-CL interaction in connexin channel gating is also available for Cx46, Cx40, and Cx26. Recent evidence indicates that the G143R mutation in the second CT-directed half of the Cx46 CL acts to strengthen CT-CL interaction (Ren et al., 2013); this results in closure of GJs and opening of HCs, in line with the findings of CT-CL effects on GJs and HCs composed of Cx43 (for details on CT-CL effects on HCs see *section II.E.5*). For Cx40, CT truncation at residue 248 resulted in the disappearance of the residual conductance (Anumonwo et al., 2001), an effect that is similar to what is found in CT-257 truncated Cx43. Additionally, the Cx43-CT was able to substitute for the Cx40-CT, indicating that heterodomain interactions between Cx40 and Cx43 (two prominent vascular connexins) are also possible (see also Bouvier et al., 2009). Experiments with CT-257 truncated Cx43 further suggest that CT-CL interaction is also involved in GJ inhibition by insulin and insulin-like growth factor (Homma et al., 1998). Additional evidence is available for GJ inhibition by v-Src, where CT truncation at residue 245 was most potent in removing GJ inhibition (Zhou et al., 1999). CT-CL interaction is also involved in HC regulation by $[Ca^{2+}]_i$ (see *section II.E.5*).

For Cx26, which has a very short CT (10 vs. 150 amino acids for Cx43), things are different. Locke et al. (2011) reported CT-Cl interactions at normal pH; upon acidification, protonated aminosulfonates like taurine interact with the CL (second CT-directed half) and thereby disrupt the CT-CL interaction (Bennett, 2011; Locke et al., 2011). Thus, opposite to Cx46, Cx43, and Cx40, for Cx26 it is rather a disruption of CT-CL interaction that acts to close the channels, both GJs and HCs.

Some final remarks to conclude this part on CT-CL interaction and pH effects. As mentioned earlier, CT-CL interactions link to fast gating transitions to the subconductance state, which stands in contrast to the fact that chemical gating is proposed to be mediated by slow gating. It needs to be added that issues concerning the identity and functions of slow and fast gates are not unequivocally set. Additionally, GJ closure with acidification is not the prerogative of CT-CL interactions; direct effects mediated by His residues have also been reported [e.g., His-95 in TM2 of Cx43 (Ek et al., 1994)]. Another possibility for GJ closure relates to the fact that protons can displace Ca^{2+} from common binding sites, thereby indirectly closing the GJs by an increase of $[Ca^{2+}]_i$ (see Peracchia, 2004 and *section II.E.5*). Of note, a low pH_i inhibits both GJs and HCs (Spray et al., 1981; Trexler et al., 1999) and there is also evidence that high pH_e promotes HC opening (Schalper et al., 2010). As always, exceptions exist and acidosis due to moderate acid load was recently reported to increase rather than suppress GJ coupling (Swietach et al., 2007).

3. Voltage-Dependent N-Terminal Tail-Linked Gating. Just as the CT plays an important role in connexin channel gating, the NT domain also plays a forefront role. It has been known from 20 years of work from the Bargiello group that the NT, which is very short (~20 amino acids for Cx26, ~22 for Cx32, and ~13 for Cx43), is involved in V_j voltage sensing (Verselis et al., 1994; Oh et al., 2000; Purnick et al., 2000). Especially position 2 as well as amino acids at the TM1/EL1 border appear to be crucial for voltage sensing in both Cx26 and Cx32 (for Cx26 crucial amino acids are Asp-2 and Lys-41 at TM1/EL1). Work from these authors demonstrated that NT-linked gating involved switching between open and a subconductance state and suggested that the NT moves toward the cytoplasm upon HC closure (Verselis et al., 1994). Moreover, it was concluded that NT movement of a single subunit was sufficient to switch to the subconductance state (Oh et al., 2000), which is in contrast to the cooperative tilting model whereby all subunits move in a concerted manner to open or close the channel (Unwin and Ennis, 1984). Later work by Maeda et al. (2009) based on 3.5 Å crystal structure of Cx26 demonstrated that the NT makes a bend into the cytoplasmic side of the HC and is kept there in a stable position by an interaction that involves Met-34 in TM1 and others in TM1, first half of EL1, and TM2 (Maeda et al., 2009; Harris and Contreras, 2014). This particular "attached" configuration of the NT is hypothesized to determine the 14 Å pore diameter of the open channel. When the voltage-sensing amino acids (especially Asp-2 and Lys-41 for Cx26) are exposed to a large junctional voltage difference, the

NT-attachment in the pore wall is destabilized, causing NT movement toward the cytoplasm and narrowing down of the pore to a subconductance state.

Both CT-CL and NT-linked gating has been proposed to be involved in gating but from different contexts that link to chemical (pH) and voltage gating, respectively. It is currently unestablished whether NT- and CT-linked gating have something in common; in that regard, the CL has been proposed as a candidate interaction target for both CT and NT (see e.g., Fig. 14 in Harris, 2001). Another question is how the voltage-sensing amino acids in NT/TM1/EL1 CT could influence CT-CL interaction. Of note, the voltage-sensing domain of the slow gate is currently unidentified. Clearly, connexin channels do not have a defined and unique voltage-sensor domain like the S4 domain of Na^+ channels, where four or more positively charged amino acids (Arg or Lys) are each separated by two non-charged amino acids (see Fig. 2 in Moreau et al., 2014). Likewise, the gates of connexin channels are not separate units clearly distinguishable from the voltage sensor (Harris and Contreras, 2014), and it looks like connexins combine their voltage sensing, gating, and perhaps also permeation conduit functions in multipurpose domains.

4. Ca^{2+}-Based Gating–Effects of Extracellular Ca^{2+}. Plasma membrane HCs not incorporated into GJs are kept in a closed state by several mechanisms that include absence of loop interactions with opposed HCs, the negative V_m, and the 1–2 mM extracellular Ca^{2+} concentration ($[Ca^{2+}]_e$) (reviewed in Fasciani et al., 2013; Harris and Contreras, 2014). When $[Ca^{2+}]_e$ falls, HCs will open as demonstrated in cardiomyocytes (Kondo et al., 2000), blood vessel endothelial cells (De Bock et al., 2012), blood cells (Eltzschig et al., 2006), and glial cells in brain slices (Torres et al., 2012). Almost all connexins display HC opening at low $[Ca^{2+}]_e$, including Cx26 and Cx30 (Lopez et al., 2013, 2016), Cx32 (Gómez-Hernández et al., 2003), Cx37 (Puljung et al., 2004), Cx40 (Allen et al., 2011), Cx43 (Bruzzone et al., 2001; Contreras et al., 2003; Ye et al., 2003; Thimm et al., 2005), Cx46 (Paul et al., 1991; Ebihara et al., 2003), and Cx50 (Zampighi et al., 1999; Beahm and Hall, 2002). In general, HCs start to open when $[Ca^{2+}]_e$ decreases below 0.5 mM, but there are substantial differences between different connexins. In addition to Ca^{2+}, Mg^{2+} also plays a role but Ca^{2+} exerts a dominant effect (Ebihara et al., 2003). Work from the Lal group with atomic force microscopy elegantly demonstrated that lowering $[Ca^{2+}]_e$ increases the Cx43 HC outer pore size (Thimm et al., 2005). In Cx32 (a liver, oligodendrocyte, and Schwann cell connexin), the high $[Ca^{2+}]_e$ closure has been traced down to Asp-169 and Asp-178 in the first half of EL2 that together form a ring of 12 Asp residues in the external vestibule of the HC (Gómez-Hernández et al., 2003); this structure is conserved in Cx30 (an astrocyte and skin connexin), Cx46, and Cx43. For Cx46, the gate that closes the channel with millimolar $[Ca^{2+}]_e$ has been reported to be situated in extracellular direction relative to the position of Leu-35 in TM1 (Pfahnl and Dahl, 1999). Recent work from the Contreras group, based on computational molecular dynamics simulations of Cx26, has demonstrated that $[Ca^{2+}]_e$ closes the HCs by disrupting intersubunit salt bridges between Asp-50 and Lys-61 located in EL1 (Lopez et al., 2016). The authors proposed that Ca^{2+} binds to Asp-50 and Glu-47 in the same region, which would form a ring of 12 negatively charged residues as suggested for Cx32 (Gómez-Hernández et al., 2003). The Lopez et al. (2016) study also suggested that the actual gate closing the channel is presumably located deeper into the channel. The Barrio group brought up evidence that, for Cx32, HC opening triggered by lowering $[Ca^{2+}]_e$ involves transitions to a subconductance state (~18 pS) and from there to a main conductance state (~90 pS), with an inverse sequence occurring upon restoration of normal $[Ca^{2+}]_e$ (reviewed in Fasciani et al., 2013). Of note, $[Ca^{2+}]_e$ may also affect voltage gating as demonstrated for Cx46 (Ebihara and Steiner, 1993; Ebihara et al., 2003; Verselis and Srinivas, 2008). In particular, lowering $[Ca^{2+}]_e$ results in the unshielding of negative charges associated with the glycocalyx outside the cell, which attracts positive charge at the inside of the membrane capacitor and acts as a depolarizing stimulus on the voltage sensor. As a result, the voltage threshold for activation is lowered, resulting in a left shift of the voltage activation curve. Moreover, De Vuyst et al. (2006), reported that hemichannel-mediated ATP release triggered by exposure of Cx32 expressing cells to divalent-free solutions ($Ca^{2\pm}$- and Mg^{2+}-free) was dependent on $[Ca^{2+}]_i$ changes, indicating that lowering $[Ca^{2+}]_e$ also influences HC opening by provoking $[Ca^{2+}]_i$ dynamics (De Vuyst et al., 2006).

5. Ca^{2+}-Based Gating–Effects of Intracellular Ca^{2+}. In contrast to $[Ca^{2+}]_e$ that only influences HCs, alterations of $[Ca^{2+}]_i$ affect both GJs and HCs. We first discuss effects on GJs and subsequently discuss more recently found effects of $[Ca^{2+}]_i$ on HCs.

a. Intracellular Ca^{2+} concentration effects on gap junctions. GJs close in response to large V_j differences and acidification, but also in response to $[Ca^{2+}]_i$ elevation. Rose and Loewenstein (1975) demonstrated more than 40 years ago that injection of Ca^{2+} in one cell of a coupled cell-pair inhibits electrical coupling between cells (Rose and Loewenstein, 1975). The closing of GJs is considered to be protective, because it will isolate the cell displaying a pathologic process, thereby avoiding GJ-mediated bystander effects on healthy neighbors. The $[Ca^{2+}]_i$ at which GJs close is rather variable between different studies and ranges from 300 nM (Lazrak and Peracchia, 1993; Crow et al., 1994; Lurtz and Louis, 2007) to several micrometers (Rose and Loewenstein, 1975; Spray et al., 1982). This may result from differences between the involved connexins or differences between cell types; it may also be linked to

the fact that global rather than microdomain $[Ca^{2+}]_i$ was measured. It furthermore appears that Ca^{2+} entry is more effective in closing GJs compared with Ca^{2+} store release (Lazrak et al., 1994). Dakin et al. (2005), making use of a photoactivated fluorescent probe, elegantly demonstrated that capacitative Ca^{2+} entry following store release was particularly potent in inhibiting junctional coupling (Dakin et al., 2005). Although Ca^{2+}-inhibition of GJs has been reported to be fast (Lazrak et al., 1994), others have reported slower kinetics sometimes in the order of minutes (Churchill et al., 2001). Gap junction closure by $[Ca^{2+}]_i$ elevation is thought to be mediated by Ca^{2+}-calmodulin signaling (reviewed in Peracchia, 2004; Lurtz and Louis, 2007). Cx32 has (at least) two calmodulin interaction sites located on the NT and CT (Torok et al., 1997), whereas Cx43 has one on its CL (Zhou et al., 2007) (Fig. 1). Ca^{2+}-triggered GJ closure can be prevented by the calmodulin inhibitor W7 (Peracchia, 1987) and by a peptide that mimics the calmodulin interaction site on the CL of Cx43 (Zhou et al., 2007) (Lys-136–Ser-158 indicated as the "CaM" domain in Fig. 1). Peracchia and coworkers (Peracchia et al., 2000; Peracchia, 2004) suggested that Ca^{2+}-calmodulin closure of Cx32 channels is mediated by a cork-plug model that involves channel pore obstruction by one of the calmodulin spherical lobes. Calmodulin may additionally act indirectly via calmodulin-dependent kinases below in *section II.E.5.b*). Finally, as remarked for pH effects, there are exceptional findings as well, in that $[Ca^{2+}]_i$ elevation or Ca^{2+}-linked signaling has been reported to increase GJ coupling in heart as well as brain (Alev et al., 2008; De Pina-Benabou et al., 2001; Delage and Délèze, 1998; Siu et al., 2016). Clearly, $[Ca^{2+}]_i$ modulation of GJs may occur via multiple intermediate signaling steps, including various kinases; as a consequence, the kinetics can be slow and the outcome on channel function diverse.

b. Intracellular Ca^{2+} concentration effects on hemichannels. Although $[Ca^{2+}]_i$ elevation mostly closes GJs, its effects on HCs link to activation of channel opening. For Cx32, De Vuyst et al. (2006) reported, based on $[Ca^{2+}]_i$ measurements and dye uptake or ATP release studies, that $[Ca^{2+}]_i$ elevation triggers HC opening (De Vuyst et al., 2006); similar findings were reported in a follow up study performed on Cx43 (De Vuyst et al., 2009). In these studies, $[Ca^{2+}]_i$ was increased by a Ca^{2+} ionophore, triggering Ca^{2+} entry, or by photoactivation of caged (inactive) IP_3 that triggers ER Ca^{2+} release. The $[Ca^{2+}]_i$ response curve was peculiar in that elevations up to 500 nM promoted HC opening, whereas $[Ca^{2+}]_i$ elevation above 500 nM resulted in a gradual disappearance of the promotive effect, giving a convex-up bell-shaped response curve with a peak at 500 nM and closure at 1 μM $[Ca^{2+}]_i$ (Fig. 2B). The closure at 1 μM $[Ca^{2+}]_i$ possibly acts as a brake to prevent excessive HC opening; a similar brake—albeit mediated by ATP—exists for Panx1 channels where ATP released via the channel inhibits the channel (Qiu and Dahl, 2009). The bell shape of HC responses to $[Ca^{2+}]_i$ elevation was also observed at the single channel level in HeLa cells overexpressing Cx43 (Wang et al., 2012a; Bol et al., 2016). In these cells $[Ca^{2+}]_i$ modulated the HC opening activity triggered by voltage steps to positive voltages (+40 mV and higher) but it did not trigger HC opening by itself; imposing $[Ca^{2+}]_i$ elevations in the absence of the electrical trigger were ineffective in opening HCs. $[Ca^{2+}]_i$ however had another interesting effect in that it lowered the voltage activation threshold for HC opening; even small $[Ca^{2+}]_i$ elevations from 50 to 200 nM lowered the voltage threshold by ~15 mV (Wang et al., 2012a). Note that $[Ca^{2+}]_e$ also affects the voltage activation of HC opening but here the activation threshold is lowered by a decrease in $[Ca^{2+}]_e$ (Contreras et al., 2003). The HC enhancing effect of moderate ($\leq$500 nM) $[Ca^{2+}]_i$ elevation has not only been observed in Cx43 expressing HeLa cells but has also been reported for acutely isolated ventricular cardiomyocytes (Wang et al., 2012a). Recent evidence obtained in mouse astrocytes furthermore demonstrates HC opening in response to $[Ca^{2+}]_i$ elevation with the membrane potential clamped at −70 mV, i.e., without associated electrical stimulation (Meunier et al., 2017); similar observations are available for mouse and pig ventricular cardiomyocytes (unpublished data). The mechanism of the $[Ca^{2+}]_i$ enhancing effect on HC opening activity has been linked to calmodulin, calmodulin-dependent kinase II, and several other factors; calmodulin-activation was particularly robust and was also effective when it was activated in a Ca^{2+}-independent manner by a Ca^{2+}-like peptide called CALP (De Vuyst et al., 2009). The disappearance of enhanced HC function at higher, above 500 nM $[Ca^{2+}]_i$ elevation (Fig. 2B) was shown to depend on actomyosin contractility that acts to disrupt intramolecular/intersubunit CT-CL interaction (Ponsaerts et al., 2010; reviewed in Ponsaerts et al., 2012). In line with this, CT-CL disruption by high (1 μM) $[Ca^{2+}]_i$ was largely prevented by the myosin II ATPase inhibitor blebbistatin (Ponsaerts et al., 2008). The location of the actomyosin interaction site on the Cx43 CT is not known but the ZO-1 site has been excluded; involvement of the Drebrin site (see Fig. 1; Ambrosi et al., 2016), which like ZO-1 links to F-actin, still needs to be verified. Collectively, this work combined with the observations of Wang et al. (2013c), demonstrated that CT interaction with the CL is necessary for Cx43 HCs to become available for opening when a trigger is present; in the absence of CT-CL interaction, HCs are unresponsive to triggers and remain in the closed state (Fig. 2A) (Wang et al., 2013a,c). CT-CL interaction involves the CT9 domain (Fig. 1) that interacts with the CL-located L2 domain, whereby the negatively charged Asp-378 and Asp-379 residues on the CT play a crucial role (D'Hondt et al., 2013).

Interestingly, supplying CT9 peptide prevented the HC closure at high (1 μM) $[Ca^{2+}]_i$ and thus removes the HC brake, most likely by directly binding to the L2 domain on the CL thereby acting as a CT substitute (Bol et al., 2016). Thus, in HCs, at least those composed of Cx43, CT-CL interaction is necessary for HCs to open, whereas the very same interaction closes the GJs, possibly by a particle-receptor scenario as explained previously (Fig. 2A). Data of Schalper et al. (2008) with fibroblast growth factor (FGF)-1 stimulation are in line with these observations; these authors reported that FGF-1 triggered Cx43 HC opening was dependent on $[Ca^{2+}]_i$ and lost upon CT truncation at position 257 (Schalper et al., 2008). It needs to be added that CT-truncated Cx43 (at Lys-258) still displays HC opening with low $[Ca^{2+}]_e$ stimulation (Kozoriz et al., 2010), indicating that the CT-CL interaction hypothesis might only be valid for triggers that come from the intracellular side such as V_m steps to positive voltages or increases in $[Ca^{2+}]_i$. Of note, exposure of cells to low $[Ca^{2+}]_e$ may provoke $[Ca^{2+}]_i$ changes that act as the actual trigger of HC opening (De Vuyst et al., 2006). Besides effects of $[Ca^{2+}]_i$ elevation on HC gating, Ca^{2+} may influence connexin trafficking as well: ER Ca^{2+} release, triggered by oxidative stress, promoted Cx43 HC opening by increasing the cell surface HC pool as determined from biotinylation experiments (Riquelme and Jiang, 2013).

Modulation of HCs and GJs in opposite directions, as observed in the context of CT-CL interaction (Fig. 2A), are not uncommon and have been reported for several conditions and signals. Also $[Ca^{2+}]_i$ distinctly influences the two channel types, because it promotes HC opening (at least when $[Ca^{2+}]_i$ is below 500 nM; see Fig. 2B) while it is well documented to inhibit GJs (exceptions exist here as well, as referred to earlier). Several other modulatory factors have opposite effects, most notably proinflammatory cytokines like tumor necrosis factor-α (TNF-α) and interleukin 1-β (IL1-β), or bacterial lipopolysaccharide (LPS), which all promote HC opening but close GJs (De Vuyst et al., 2007; Retamal et al., 2007a; Orellana et al., 2009); metabolic inhibition has a similar effect (Contreras et al., 2002). Along the same line, arachidonic acid inhibits GJs but promotes HC opening (Contreras et al., 2002; De Vuyst et al., 2007, 2009). By contrast, acidification inhibits both GJs and HCs (Spray et al., 1981; Trexler et al., 1999), a remarkable observation because low pH_i closure of GJs is supposed to be mediated by CT-CL interaction while this very same interaction is expected to promote HC opening. However, Wang et al. (2012a) reported that Cx43 HC closure at low pH_i disappears under conditions of strong cytoplasmic Ca^{2+} buffering with 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (commonly known as BAPTA), indicating that low pH_i closure depends on $[Ca^{2+}]_i$ elevation (Wang et al., 2012a). Another possibility is that acidification has more direct effects on Cx43 that do not depend on CT-CL interaction.

6. Other Gating Effects. Several other HC opening triggers have been reported, but not all of them have been characterized at the electrophysiological level. Retamal et al. (2007b) reported that Cx43 HCs open in response to intracellularly applied reducing conditions making use of glutathione or dithiothreitol (Retamal et al., 2007b), an effect that was exercised at the level of channel gating. The latter experiments were done under normoxic conditions; under ischemic conditions (mimicked by metabolic inhibitors) astrocytic HCs also opened, but this was linked to an increase of the plasma membrane HC pool. The resulting increase of HC function was mediated by *S*-nitrosylation, possibly by NO acting at CT-located Cys residues, and inhibited by reducing agents (Retamal et al., 2006; reviewed in Sáez et al., 2005). Interestingly, NO is an HC permeant that may also activate HC opening (Retamal et al., 2006; Figueroa et al., 2013), as is the case for Ca^{2+} [Ca^{2+} permeates as well as activates channel opening by elevating $[Ca^{2+}]_i$; NO can freely diffuse over the plasma membrane but when open HCs are available, they may facilitate NO diffusion (Figueroa et al., 2013)]. Work from the Lal group demonstrated Cx32 and Cx43 HC opening in response to H_2O_2-induced generation of reactive oxygen species (ROS) that appeared to involve V_m changes (Ramachandran et al., 2007). Carbon monoxide (CO) inhibits Cx43- and Cx46-based HCs independently of CT Cys residues, and the effect disappears by application of extracellularly acting reducing agents (León-Paravic et al., 2014). Effects of phosphorylation on gating of GJs/HCs are addressed *section II.F*.

F. Connexin Posttranslational Modifications

Many different connexin posttranslational modifications have been reported, including phosphorylation, ubiquitination, sumoylation, *S*-nitrosylation, palmitoylation, hydroxylation, acetylation, methylation, and γ-carboxyglutamation. However, the evidence supporting their prevalence and biologic significance varies considerably. Evidence that can support the existence and importance of posttranslation modifications includes detection of the modification using multiple analytical techniques, finding the modification in cells and tissues and observation of a biologic effect if that posttranslational modification is prevented. For example, detection of a posttranslational modification via direct incorporation of a radioactive element that is part of the posttranslational modification, detection via mass spectrometry, and/or detection via an antibody to the modification in connexin purified from cells would be convincing evidence that a posttranslational modification can occur. However, detection via a single method can be potentially misleading for several reasons. For example, amino acid modifications can be

introduced during protein purification steps and then they are easily detected via high sensitivity mass spectrometers currently in use today. In vitro phosphorylation of a connexin, often just the CT region combined with a purified kinase, can indicate a phosphorylation event is possible, but without evidence that it happens in cells, the result by itself is not particularly important. Artificial introduction of a modification can also occur via overexpression of the substrate or the enzyme that catalyzes the modification in cells. Because all chemical reactions even within cells depend on the concentrations of the reactants, they may be possible under artificial conditions but not occur significantly in vivo. Therefore, multiple lines of evidence supporting the modification including its presence in vivo gives one more confidence that a modification was not artificially introduced. Furthermore, if modulation of the modification in vivo changes the biologic properties of GJ communication or other connexin characteristics (e.g., changes in interacting proteins or connexin localization) that would be good evidence of biologic relevance. For example, good evidence that a posttranslational modification is biologically meaningful might be derived from detection of the posttranslational modification by multiple methods in vivo (e.g., immunoprecipitation and detection via mass spectrometry or isotope incorporation and detection in cells via an antibody to the modification). Biologic relevance could be shown via modulation of the extent of modification through application of a targeted stimulus that elicits a physiologic change in connexin properties and elimination of the modulatory effect of the phosphorylation event when the specific connexin amino acid is mutated so it cannot be modified.

1. Phosphorylation. The best documented connexin posttranslational modification is phosphorylation. Connexin phosphorylation has been reported to affect connexin half-life, protein trafficking, incorporation into a GJ, GJ size, channel gating, and protein turnover (for recent reviews, see Pogoda et al., 2016; Solan and Lampe, 2016). Furthermore, wounding, ischemia, and other tissue insults have been shown to change connexin phosphorylation. Knock-in mice where three Ser to Ala or Ser to Glu point mutations were introduced in the gene for Cx43 at sites phosphorylated by casein kinase 1 (CK1) were highly susceptible or resistant to inducible arrhythmias, respectively (Remo et al., 2011). Knock-in mice expressing Cx43 with mitogen-activated protein kinase (MAPK) phosphorylation sites mutated to Ala showed reduced proliferation during arteriole injury and reduced neointima formation (Johnstone et al., 2012). Knock-in mice with Cx43 containing a Ser to Ala mutation at a protein kinase C (PKC) site were unresponsive to sphingosine-1-phosphate cardioprotective effects upon ischemia reperfusion injury (Morel et al., 2016). These results combined with those showing that these residues are phosphorylated in vivo essentially prove that connexin phosphorylation plays key roles in the physiologic response to injury.

Many connexins isolated from cellular preparations have been shown to incorporate radioactive phosphorous; demonstrate phosphatase-dependent shifts in mobility in SDS-PAGE; exhibit charge/mass ratios via mass spectrometry that are consistent with phosphorylation; and/or yield phospho-Ser, -Thr, and/or -Tyr in amino acid and peptide analyses. However, as for other posttranslational modifications, the extent of the evidence varies by connexin. There is at least one report of phosphorylation (discussed below) of most of the connexin α group members, including Cx37 (Morel et al., 2010), Cx40, Cx43, Cx46, and Cx50 and for Cx36, Cx45, and Cx47 (May et al., 2013), that belong to the γ group (α,β or γ group assignations; see Cruciani and Mikalsen, 2006). Phosphorylation of other connexin group members, including Cx29 (Wiśniewski et al., 2010), Cx31 (Diestel et al., 2004), Cx31.9 (Nielsen and Kumar, 2003), and Cx32 (discussed below), has also been reported, but little biologic data supporting their biologic relevance exist. At least two connexin proteins (Cx26 and Cx33) have been reported to be not phosphorylated. In the next several paragraphs, we discuss the connexins for which significant data exist.

a. Cx43 phosphorylation. The most well-studied and characterized phosphoconnexin is Cx43. At least 19 of the 26 Ser and 4 of the 6 Tyr in the CT region of Cx43 have been identified as kinase substrates, and there has been some progress in the characterization of the network of kinases that phosphorylate Cx43 (phosphorylation sites are illustrated in Fig. 1 and major effects are summarized in Table 1). Of course, the level of evidence supporting these sites and the kinases involved varies considerably. Sites with high confidence include those phosphorylated by MAPK family members, PKC (especially δ and ε), Src, Akt, and CK1. MAPK3/MAPK1 (ERK1/2) are known to phosphorylate Ser-255, Ser-279, and Ser-282 in vitro (Warn-Cramer et al., 1996); these sites are phosphorylated in cells when MAPK is activated and GJ channel properties are modulated in a manner that is abrogated when the sites are mutated to Ala (Warn-Cramer et al., 1998). MAPK7 can also phosphorylate Ser-255 (Cameron et al., 2003), and Ser-262 has been identified as a substrate for CDK1 (Kanemitsu et al., 1998). Phosphospecific antibodies have been created for all of these sites and they react with Cx43 in Western blots and via immunofluorescence in the expected manner when MAPK is activated or inhibited. Although the exact sites and stoichiometry of the phosphorylation events are not clear, in vitro phosphorylation of Cx43 with purified CK1 yields phosphorylation at Ser-325, Ser-328, and Ser-330, and CK1 activation increases their phosphorylation, whereas inhibition decreases phosphorylation (Table 1). Assaying with a phosphospecific antibody for these sites reports decreased signal during hypoxia, and

TABLE 1
Residues, kinases, and effects of Cx43 phosphorylation

Assembly/Disassembly Residues	Kinases	Direction of Effect	References
Ser-325,-328,-330	CK1	GJs ↑	Cooper and Lampe (2002)
Ser-364,-365		GJs ↑	TenBroek et al. (2001), Shah et al. (2002), Yogo et al. (2002), Solan et al. (2007)
Ser-369, -373	Akt/PKB	GJs ↑	Shah et al. (2002), Yogo et al. (2002), Park et al. (2007), Dunn and Lampe, (2014)
		HCs ↑	Salas et al. (2015)
Ser-372		GJs ↑	Sáez et al. (1997), Yogo et al. (2002), Park et al. (2007), Dunn and Lampe (2014)
Ser-368	PKC	GJs ↓, ↑	Sáez et al. (1997), Axelsen et al. (2006), Procida et al. (2009)
Tyr-247, -265, -313	Src, Tyk2	GJs ↓	Lin et al. (2001), Ballif et al. (2008), Bonnette et al. (2010), Solan and Lampe (2014), Li et al. (2016)
Gating residues			
Ser-255, -279, -282	MAPK	GJs ↓	Warn-Cramer et al. (1996)
Ser-262	p34cdc2, MAPK	GJs ↓	Kanemitsu et al. (1998), Lampe et al. (1998), Doble et al. (2004)
Ser-296, 297, 306		GJs ↑	Axelsen et al. (2006), Procida et al. (2009)
Ser-368	PKC	GJs ↓	Sáez et al. (1997), Axelsen et al. (2006), Procida et al. (2009)
		HCs ↓	Bao et al. (2004, 2007), De Vuyst et al. (2007), Hawat and Baroudi (2008)

mutation of these sites affect the development of cardiac arrhythmia (Remo et al., 2011). Tyr-247 and Tyr-265 of Cx43 are known Src substrates and Tyr-301 and Tyr-313 were identified as phosphorylated in general MS/MS-based screens for phosphotyrosine-containing proteins (Ballif et al., 2008; Bonnette et al., 2010). A recent report indicates that Tyk2 can phosphorylate the same Tyr residues as Src (Li et al., 2016). The last 19 amino acids of Cx43 contain three sets of double Ser (Ser-364/Ser-365, Ser-368/Ser-369, Ser-372/Ser-373) that all have been reported to be phosphoacceptors (e.g., Yogo et al., 2002). Ser-368 is a very well-documented substrate for classic PKCs, and early creation of a phosphospecific antibody for this site has led to dozens of reports of Ser-368 modulation in response to various cellular treatments and conditions. Akt phosphorylates Cx43 primarily at Ser-373 and secondarily at Ser-369 in vitro (Park et al., 2007), and the binding of a phosphospecific antibody for phosphorylated Ser-373 is decreased when Akt is inhibited in cells (Dunn and Lampe, 2014). Ser-364 (TenBroek et al., 2001) and Ser-365 (Solan et al., 2007) are also known to be phosphorylated particularly under conditions where PKA is activated, but the kinase(s) that actually phosphorylate these residues is less clear as it is a poor substrate for direct phosphorylation by PKA (TenBroek et al., 2001; Shah et al., 2002).

There are other documented Cx43 phosphorylation sites for which the kinase responsible is less clear. Ser-262 has been reported to be phosphorylated in response to PKC activation (Doble et al., 2004; Srisakuldee et al., 2014), but it was not a major substrate for purified classic PKCs (Sáez et al., 1997; Lampe et al., 2000), raising the question of whether PKC activation could cause activation of other kinases, potentially proline-directed kinases, because there is a Pro C terminus to Ser-262 fitting the consensus sequence and it is a known substrate for a proline-directed kinase (Kanemitsu et al., 1998). A similar issue was observed for Src activation, which subsequently leads to MAPK and PKC activation and phosphorylation at Ser-255, Ser-262, Ser-279, Ser-282, Ser-368, Tyr-247, and Tyr-265 (Solan and Lampe, 2008), potentially leading to confusion as to which sites and kinases actually are responsible for specific effects (Lin et al., 2001; Mitra et al., 2012). There are other reported sites for which we have less information, like Ser-296, Ser-297, Ser-306, and Ser-314 (Procida et al., 2009), which need to be further investigated. Nonetheless, we know much more about Cx43 regulation via phosphorylation compared with the other connexins.

b. Cx32 phosphorylation. Cx32 was shown to be phosphorylated by metabolic labeling of hepatocytes, and phosphorylation levels were shown to increase with 8-bromo-cAMP (Sáez et al., 1986), forskolin, or phorbol ester treatment (Sáez et al., 1990). Ser-233 appeared to be a substrate for both PKA and PKC, and its phosphorylation level increased when the kinases were activated in cultured cells (Sáez et al., 1990). Epidermal growth factor receptor tyrosine kinase may also be involved in phosphorylation of Cx32 on tyrosine residues (Díez et al., 1998). However, the physiologic effects of all of the Cx32 phosphorylation events need to be studied further to better understand their biologic implications.

c. Cx36 phosphorylation. There has been a great deal of interest in the regulation of Cx36, because it has been found to be expressed in neurons as well as in dendrites of AII amacrine cells of the retina. Cx36 was shown to be a phosphoprotein by metabolic labeling and mass spectrometry (Urschel et al., 2006). Phosphospecific antibodies to PKA substrates Ser-110 and Ser-276 in Bass (Ser-293 in mammals) showed that large GJs in the inner plexiform layer showed higher levels of

phosphorylation in dark-adapted and reduced levels in light-adapted retina (Kothmann et al., 2007). Dopamine-driven, PKA-dependent uncoupling of the AII amacrine cell network occurs via PKA activation of protein phosphatase 2A and subsequent dephosphorylation of Cx36 (Kothmann et al., 2009).

d. Cx40 phosphorylation. Cx40 was shown to be phosphorylated via metabolic labeling (Traub et al., 1994). 8-Br-cAMP addition to Cx40-transfected cells resulted in a SDS-PAGE mobility shift in Cx40 and increased macroscopic GJ conductance between cell pairs (van Rijen et al., 2000). LPS or hypoxia and reoxygenation treatment of microvascular endothelial cells led to a reduction in Cx40 phosphorylation and electrical coupling between cells that was dependent on PKA (Bolon et al., 2008).

e. Cx45 phosphorylation. Cx45 has been convincingly shown to be phosphorylated via metabolic incorporation and phosphatase-dependent SDS-PAGE mobility shifts at primarily serine residues (Butterweck et al., 1994; Darrow et al., 1995), with less phosphotyrosine and phosphothreonine also being reported (Hertlein et al., 1998). Serines present within residues 374–393 were responsible for 89% of the phosphorylation and, in particular, Ser-381, -382, -384, and/or -385 were found to be important in regulating Cx45 stability, because mutation of different combinations of these reduced the half-life of the mutant version to 50% of wild-type Cx45 (Hertlein et al., 1998). Treatment of cells expressing Cx45 with 8-Br-cAMP or pervanadate increased Cx45 phosphorylation levels and decreased GJ conduction (van Veen et al., 2000).

f. Cx46 and Cx50 phosphorylation. These connexins are usually coexpressed in tissues, and several reports examined the phosphorylation status of both proteins so they are covered here together. Both migration shifts (Pelletier et al., 2015) and immunoprecipitation followed by mass spectrometry (Lin et al., 2004) have shown Cx46 and Cx50 to be phosphorylated at 9 and 18 serine residues, respectively (Wang and Schey, 2009). PKA phosphorylated Ser-395 of Cx50 and mutation of this residue to alanine attenuated increases in dye coupling and uptake caused by PLA activation (Liu et al., 2011). Phosphorylation of Cx50 can accelerate turnover (Yin et al., 2000) and susceptibility to caspase-3-like protease cleavage (Yin et al., 2001).

2. Connexin Ubiquitination. Protein ubiquitination controls many aspects of cellular function by targeting substrates for degradation via the proteasome and through ubiquitin-dependent protein-protein interactions. There has been at least one report that Cx26 (Xiao et al., 2014), Cx32 (Kelly et al., 2007), and Cx40 (Gemel et al., 2014b) can be ubiquitinated, and there are many reports for Cx43. Furthermore, inhibition of the proteasome dramatically increases the levels of Cx40 (Gemel et al., 2014b) and Cx43 present within GJs (VanSlyke and Musil, 2005; Dunn and Lampe, 2014). However, ubiquitin signaling is complex and there are many inconsistencies in the literature, potentially due to the variety of cellular systems under study that complicate our understanding of its role in connexin biology. For example, different reports indicate that Cx43 is mono-ubiquitinated (Leithe and Rivedal, 2004; Girão et al., 2009) or it is poly-ubiquitinated (Laing and Beyer, 1995; Laing et al., 1997; Ribeiro-Rodrigues et al., 2014; Martins-Marques et al., 2015b) and that it can preferentially be degraded by the proteasome or the lysosome. Given that the biologic consequences of mono- versus poly-ubiquitination are potentially dramatically different (i.e., resulting in putative proteasomal degradation or activity regulation), these inconsistencies need some resolution. Most of these studies used cell lines where the ubiquitin is epitope or 6xHis tagged and overexpressed. Also, most used a Cx43 antibody to immunoprecipitate Cx43 and then cut the blot in two and probed the top half with antiubiquitin to show a ladder or smear of signal and the lower half with anti-Cx43. The problem with this approach is that the levels and exposures of the two halves are not comparable and any protein that coprecipitates with Cx43 might be the actual ubiquitin acceptor rather than Cx43. In studies where estimates of the level of the putative ubiquitinated Cx43 isoform were made, it appeared to be much less than 1% of the total Cx43 comigrated with what might be an ubiquitinated isoform under conditions that should favor its accumulation (Girão et al., 2009; Chen et al., 2012b). Furthermore, conversion of all Lys in Cx43 to Arg to eliminate ubiquitination did not affect the accumulation of Cx43 in GJs in response to proteasomal inhibitors (Dunn et al., 2012) or during ER-localized degradation (Su et al., 2010). In fact, two other Cx43-interacting proteins that are ubiquitin substrates or ubiquitin associated, Akt (Dunn and Lampe, 2014) and CIP75 (Su et al., 2010), were found to regulate these processes in ubiquitin-dependent processes. Therefore, caution should be exercised when ubiquitin-related data are interpreted. On the other hand, a fusion protein of Cx43 and ubiquitin had a shorter half-life than wild-type Cx43 (Catarino et al., 2011). Clearly the ubiquitin system regulates connexin distribution. However, given its low levels and lack of a role in GJ size regulation and ER-associated degradation, further research needs to be performed to define the biologic function of Cx43 and connexin ubiquitination in general. One possible explanation consistent with at least some of these results is that ubiquitination of Cx43 does occur but only under specific situations such as autophagy (further discussed *section II.F.4* below).

3. Other Potential Connexin Posttranslational Modifications. The isoelectric point of Cx43 is lower than predicted from its sequence in a manner thought to be independent of phosphorylation (Stockert et al., 1999), suggesting other modifications might be possible.

A handful of reports indicate that connexins (e.g., Cx26 and Cx43) are also posttranslationally modified by *S*-nitrosylation (Retamal et al., 2006), SUMOylation (Kjenseth et al., 2012), acetylation (Colussi et al., 2011), palmitoylation, hydroxylation, methylation, and γ-carboxyglutamination. Methylation, acetylation, hydroxylation, palmitoylation, and γ-carboxyglutamination of Cx26 were reported as possible posttranslational modifications in an MS-based study of purified Cx26 (Locke et al., 2009). Although intriguing, as pointed out previously (Chen et al., 2013), this MS study used a limited search strategy based on accurate masses expected for Cx26 fragments rather than MS/MS analysis using broad search strategies, and this could have easily generated false discovery. Furthermore, the highly sensitive mass spectrometers in use today can generate false-positive posttranslational modification detection at many steps from protein purification (e.g., silver staining of gels) to MS data peak assignment (Larsen et al., 2006; Kim et al., 2016a). Therefore, confirmation via other techniques and studies will be necessary for all of these modifications, and if confirmed, understanding their biologic effect will become important.

4. Regulation of the Connexin Life Cycle via Posttranslational Modifications.

a. Connexin protein half-life. Although GJs appear as complex structures and can be difficult to disrupt during biochemical isolation, most connexin proteins have been shown to have short half-lives (1–5 hours), both in vivo and in cell culture (Crow et al., 1990; Musil et al., 1990; Laird et al., 1991; Lampe, 1994; Darrow et al., 1995; Beardslee et al., 1998; Hertlein et al., 1998; Lauf et al., 2002; Laird, 2006, 2010). However, the half-life of Cx30 (Kelly et al., 2015) and some isoforms of Cx50 (Jiang and Goodenough, 1998; Berthoud et al., 1999) appear to be much longer and more similar to typical integral membrane proteins (Chu and Doyle, 1985; Hare and Taylor, 1991). Why a cell would turnover connexin proteins so rapidly is not obvious. A short half-life with extensive regulation could allow the cell to control exquisitely processes dependent on connexin-binding protein interactions, HC function, or GJ communication. At least one region of Cx43 can control turnover, because it contains two putative Tyr-based sorting signals [Yxxϕ; where ϕ = hydrophobic (Fong et al., 2013)], including the key sequence Y^{286}KLV (see Fig. 1), which upon Val to Asp mutation displays a threefold increased protein half-life (Thomas et al., 2003). As integral membrane proteins, connexins are synthesized in the ER but they lack a canonical membrane signal sequence and at least Cx43 does not oligomerize into a hexameric HC until reaching the trans-Golgi network (Musil and Goodenough, 1993). This delay in multimerization is hypothesized to provide a quality control step, because GJ assembly can be downregulated through endoplasmic reticulum-associated degradation during conditions of cellular stress (VanSlyke and Musil, 2002; Su et al., 2010).

b. Regulation of connexin export to the plasma membrane and gap junction assembly. Live cell and other imaging techniques show multiple regulatory aspects of Cx26, Cx32, and Cx43 transport to the plasma membrane, including microtubule-based vesicle transport (Martin et al., 2000; Johnson et al., 2002; Lauf et al., 2002; Shaw et al., 2007). Imaging studies have also shown that Cx43 can move from the plasma membrane into the periphery of a larger plaque; thus plaques grow by adding channels to the outside, and the oldest proteins are found in the center of the plaque where they get selectively turned over (Gaietta et al., 2002). Live cell imaging techniques also show Cx43 is highly motile with dynamic interactions with the cytoskeleton and events at GJs (e.g., Jordan et al., 1999, 2001; Martin et al., 2001; Lauf et al., 2002; Murray et al., 2004; Fiorini et al., 2008; Solan and Lampe, 2016). Phosphorylation of Cx43 can occur within 15 minutes of synthesis (Crow et al., 1990), and several kinases have been reported to regulate the assembly of GJs. When examined via Western immunoblot, many connexins demonstrate a phosphorylation-dependent reduction in SDS-PAGE mobility. Cx43 can often show three prominent bands sometimes labeled as P0, P1, and P2. Antibodies to Cx43 phosphorylated at Ser-365 show the P1 and P2 phosphoisoforms, which occur during the transition from the cytoplasm to the plasma membrane (Solan et al., 2007; Sosinsky et al., 2007). Furthermore, Ser-365 phosphorylation plays a "gatekeeper" role by preventing downregulation of GJ communication by subsequent Cx43 phosphorylation at Ser-368 (Solan et al., 2007). Several studies show that activation of cAMP-dependent protein kinase (PKA) can stimulate Cx43 trafficking to the plasma membrane (Atkinson et al., 1995; Burghardt et al., 1995), resulting in enhanced GJ assembly (see Table 1) and increased phosphorylation at many of the last six serine residues in Cx43 (TenBroek et al., 2001; Yogo et al., 2002,2006; Solan et al., 2007). CK1 (Table 1) phosphorylates Cx43 on some combination of residues Ser-325, Ser-328, and/or Ser-330 during the transition of Cx43 from the plasma membrane into the GJ (Cooper and Lampe, 2002), and a phosphospecific antibody to this region recognizes only Cx43 present in the GJ and the P2 form of isoform Cx43 (Cooper and Lampe, 2002). Akt can phosphorylate Cx43 primarily at Ser-373 (see Table 1) and this step allows GJs to grow in size via a reduced interaction with ZO-1 (Dunn and Lampe, 2014), which eliminates the well-documented ability of ZO-1 to restrict GJ size (Hunter et al., 2005; Rhett et al., 2011). Although Cx43 GJ assembly is clearly regulated by a series of kinases that can fine tune GJ communication, our understanding of how GJ assembly is regulated for the other connexins is much less well understood.

c. Regulation of gap junction turnover. Although we understand many aspects of GJ assembly, particularly for Cx43, and connexin protein turnover can easily be examined using pulse-chase methods, several aspects of GJ turnover remain unclear. For example, live cell imaging studies show that a single GJ can be relatively stable for hours but a neighboring one can be rapidly dispersed (e.g., Solan and Lampe, 2016). Proteasomal inhibitors can stabilize Cx43-containing GJs and make them larger, but they usually only have a mild effect on the total level of Cx43 (Lampe et al., 1998; Qin et al., 2003). However, lysosomal inhibitors can increase Cx43 protein levels, but the protein appears to build up in cytoplasmic membranes (Qin et al., 2003; Berthoud et al., 2004). Internalized annular junctions (connexosomes) (Severs et al., 1989; Jordan et al., 2001; Laird, 2006; Leithe et al., 2006; Piehl et al., 2007; Fong et al., 2012; Johnson et al., 2013; Nickel et al., 2013) are apparent in some cell types and in cells undergoing autophagy, where they colocalize with the clathrin-adapter proteins disabled2 (Piehl et al., 2007), Atg14 and 9 (Bejarano et al., 2014), and the autophagosome membrane protein LC3 (Hesketh et al., 2010; Fong et al., 2012). Autophagy and degradation of multiple connexin isoforms have been linked (Iyyathurai et al., 2016). However, it is unclear whether this mechanism might account for most/all of GJ turnover or whether it occurs only in specialized circumstances such as nutrient deprivation. Part of the issue is that different treatments that affect clathrin-mediated and other internalization processes have only partial effects, making firm conclusions more difficult. Clearly more research is needed to understand whether multiple mechanisms for GJ turnover exist in vivo.

Although our knowledge of the mechanisms involved in GJ turnover is incomplete, we know that epidermal growth factor, 12-*O*-tetradecanoylphorbol acetate, Src activation, wounding, and extracellular ATP (Kanemitsu and Lau, 1993; Ruch et al., 2001; Schwiebert and Zsembery, 2003; Rivedal and Leithe, 2005; Chang et al., 2008; Dunn and Lampe, 2014) lead to Cx43 phosphorylation and loss of GJs. PKC (Lampe, 1994; Solan et al., 2003; Richards et al., 2004), MAPK (Johnstone et al., 2012), Src (Solan and Lampe, 2008), and Akt (Dunn et al., 2012) probably play at least some role in the regulation of Cx43 turnover. A kinase program that spatiotemporally activates Cx43 GJ turnover via sequential phosphorylation by Akt, MAPK, Src, and PKC in response to growth factors, wounding, and other stimuli has been proposed (Solan and Lampe, 2016). In this model, an Akt-mediated transient increase in GJ size depletes the nonjunctional Cx43 by rapid incorporation into a GJ, and the resulting larger GJ potentially reduces the energetics of annular junction formation by enabling membrane curvature during internalization. Src can clearly play a role, because its inhibition via PP2 treatment blocks growth factor-induced GJ turnover (Spinella et al., 2003; Gilleron et al., 2008). For many years it has been known that v-Src activity can downregulate GJ communication (Atkinson et al., 1981; Azarnia et al., 1988; Menko and Boettiger, 1988) coincident with an increase in tyrosine phosphorylation on Cx43 (Crow et al., 1990; Swenson et al., 1990). In LA25 cells that express active v-Src, Cx43 residues Tyr-247 (Src), Tyr-265 (Src), Ser-255 (MAPK), Ser-262 (MAPK), Ser-279/282 (MAPK), and Ser-368 (PKC) are all phosphorylated indicating co-activation of MAPK and PKC upon Src activation (Solan and Lampe, 2008). Phosphorylated Tyr-247 appeared to be preferentially present in larger GJ plaques (Solan and Lampe, 2014). Whether this distinct phosphoTyr-247 staining could potentially mark a portion of the GJ to facilitate interaction with components of the endocytic system is unknown. Src phosphorylation of Cx43 may trigger GJ endocytosis like it does with the NMDA receptor GluN3A (Chowdhury et al., 2013). There may also be three-way crosstalk regulation involved, because both ZO-1 and Src can bind to the CT region of Cx43 and they can bind to each other as well (Sorgen et al., 2004; Gilleron et al., 2008; Kieken et al., 2009), whereas Akt phosphorylation at Ser-373 inhibits ZO-1 binding to Cx43 (Dunn and Lampe, 2014). Furthermore, the GJ blocker glycyrrhetinic acid causes GJs to adopt a looser packing arrangement (Goldberg et al., 1996) in a process that involves Src binding (Chung et al., 2007) and that leads to disruption of Cx43-ZO-1 interaction (Gilleron et al., 2008).

d. Phosphorylation effects on connexin channel gating. Phosphorylation effects on connexin channel function have been amply documented based on dye transfer studies making use of various channel-permeant fluorescent molecules. The data obtained with such approaches can be interpreted in several ways, because changes in dye transfer can be caused by alterations in the number of channels available, in the permeability properties of the channels, or in the channel gating characteristics. More fundamental insights into how phosphorylation alters GJ/HC function necessitates electrophysiological analysis at the single channel level, allowing the resolution of effects on open probability and single channel conductance, including main and residual conductance states. Only a few reports, reviewed in Moreno and Lau (2007), are available that have documented phosphorylation effects at a detailed unitary channel activity level. Kwak et al. (1995b) showed that cGMP-dependent Cx43 phosphorylation at Ser-259 (present in rat but not in human) reduced the GJ macroscopic conductance by decreasing the single channel main open state to a state with a lower conduction (Kwak et al., 1995b). Follow up work demonstrated distinct effects of phosphorylation by PKA, PKC, and PKG on different connexins (Cx26, Cx43, and Cx45) based on their biophysical single channel signature (Kwak et al., 1995a). Lampe et al.

(2000) demonstrated that PKC phosphorylation at Ser-368 inhibits Cx43 GJs by shifting the unitary event activity in conductance histograms from 100 pS centered events, corresponding to the main open state, to 50–60 pS events that correspond to a residual subconductance state; this work was based on comparing channel event activities in Cx43 wild type with those in a Cx43-S368A phosphodead mutant (Lampe et al., 2000). However, PKC has also been reported to increase the open probability of Cx43 GJs (Kwak et al., 1995c; Kwak and Jongsma, 1996), making the net effect on electrical current less clear. Bao et al. (2007) demonstrated that PKC phosphorylation, in addition to the effects on gating, also alters the size selectivity as suggested by sucrose permeation experiments (Bao et al., 2007).

For v-Src, Cottrell et al. (2003) reported that GJ inhibition was not mediated by alterations in the unitary conductances; this work was based on comparing event activities in Cx43 wild type with those in cells expressing Cx43-Y247F,Y265F double mutant versions (Cottrell et al., 2003). Based on the fact that v-Src did not change the size of GJ plaques, suggesting that the number of GJ channels was not affected (Atkinson et al., 1986; Lin et al., 2001), the most probable effect of v-Src appeared to reside at the level of GJ channel open probability (Moreno and Lau, 2007). Further dye transfer studies by Cottrell et al. (2003) demonstrated that GJ channel permeability for Lucifer yellow and [2-(4-nitro-2,1,3-benzoxadiazol-7-yl)aminoethyl]trimethylammonium was also affected by v-Src phosphorylation at Tyr-247 and Tyr-265; the effects were stronger at the level of dye transfer than at the level of current inhibition, which looks similar to the PKC effects. Interestingly, CT truncation at residue 245, just two positions in NT direction relative to Tyr-247, removes GJ inhibition by v-Src (Zhou et al., 1999), making it possible that v-Src inhibition of GJs is the consequence of CT-CL particle-receptor (ball and chain) interaction (see *section II.E.2*); others have also linked v-Src to CT-CL interaction (Lau, 2005). Zhou et al. (1999) suggested that v-Src effects are indirect and mediated by MAPK effects at Ser-255, Ser-279, and Ser-282 (Warn-Cramer et al., 1998). In line with this, Cottrell et al. (2003) found that epidermal growth factor-activation of MAPK resulted in GJ inhibition that was not associated with alterations in the conductance states, as observed for v-Src; these authors furthermore demonstrated that MAPK-inhibition of GJs was absent in Cx43-S255A,279A,282A mutants. Of note, MAPK effects are fast (Kim et al., 1999) and have also been linked to CT-CL interaction (Harris, 2001).

Little is known about phosphorylation effects on the gating of channels composed of connexins other than Cx43. Sáez et al. (1986) reported that cAMP-stimulated phosphorylation of Cx32 induced a higher conductance state (Sáez et al., 1986). Similarly, van Rijen et al. (2000) reported that PKA activation, a well-known activator of GJ coupling downstream of cAMP, promoted Cx40 coupling by favoring a higher conductance state (van Rijen et al., 2000). Van Veen et al. (2000) demonstrated that pervanadate, which activates Tyr kinases, inhibited Cx45-based GJs by an effect that was concluded, based on exclusion of other possibilities, to decrease open probability (van Veen et al., 2000).

The same paucity of data applies to effects of phosphorylation at the level of gating of HCs. Several papers have suggested that MAPK family members may promote Cx43 HC opening (Schalper et al., 2008; De Vuyst et al., 2009; Avendano et al., 2015), but others have reported opposite effects (Kim et al., 1999; Riquelme et al., 2015). These results were based on indirect measures of HC function, mostly dye uptake or ATP release assays. Dye uptake studies also demonstrated that Akt/PKB, activated by metabolic inhibition, increased Cx43 HC function by increasing the HC pool in the plasma membrane as judged from biotinylation experiments (Salas et al., 2015). For PKC, all available data seem to point to inhibition of HC activity (Bao et al., 2004, 2007; De Vuyst et al., 2007); Hawat and Baroudi (2008) confirmed this at the electrophysiological macroscopic conductance level and further demonstrated that PKC_{ε} had more potent effects than PKC_{β} or PKC_{δ} (Hawat and Baroudi, 2008). Clearly, more work is necessary here to resolve HC modulation by various kinases at the unitary current level and to determine how it affects gating and whether this relates to CT- or NT-linked conformational changes. Table 1 summarizes the effects of various kinases and target residues.

III. Pharmacological Modulation of Connexin Channels

The pharmacology of connexin channels is complex. First of all, specific inhibitors like those available for Na^+, Ca^{2+}, and K^+ channels are not available. Second, most substances inhibit both GJs and HCs, except for some substances that are discussed below. Third, almost all inhibitors act in the micromolar range, not in the nanomolar range. Pharmacological modulation of GJs/HCs can, in principle, be achieved in various ways acting at distinct levels, including 1) connexin biosynthesis/expression, 2) connexin trafficking and HC assembly, 3) docking of HCs and formation of GJs, 4) formation of GJ plaques, 5) GJ disassembly by internalization and degradation, 6) the number of channels (by mechanisms related to 1 to 5), 7) channel gating, and 8) pore block or alterations in channel permeability when pore block is incomplete. Second messengers, posttranslational modifications, ions like Ca^{2+}, H^+, and membrane potential all act at least at one of these various levels, as described in the preceding sections. Several pharmacological agents may act at these different levels, either directly or indirectly by influencing intracellular signaling cascades (Table 2;

reviewed in Dhein, 2004; Salameh and Dhein, 2005). For example, the fatty acid oleic acid inhibits GJ channels via PKC phosphorylation of Ser-368 (Huang et al., 2004). Lindane, a carcinogenic insecticide, inhibits GJs by MAPK activation that promotes the displacement of membrane-located Cx43 to the intracellular compartment (Mograbi et al., 2003). Tedisamil, a class III antiarrhythmic agent, promotes GJ conductance by acting on PKA (De Mello and Thormahlen, 1999).

A. Connexin Channel Inhibitors

Several chemical classes of connexin channel inhibiting substances exist (reviewed in Salameh and Dhein, 2005; Bodendiek and Raman, 2010; Verselis and Srinivas, 2013). The best known and widely used substance in experimental work is carbenoxolone, a derivative of the licorice-derived glycyrrhetinic acid, which has better water solubility compared with 18-α-glycyrrhetinic acid and 18-β-glycyrrhetinic acid; carbenoxolone inhibits GJs with an IC_{50} in the range of ~50 μM (see Table 1 in Spray et al., 2006). The mechanism of action of this family of licorice-based molecules on connexin channels is poorly defined. 18-α-Glycyrrhetinic acid has been reported to inhibit connexin synthesis (by decreasing transcription) and/or to promote connexin turnover at concentrations above 20 μM (Guo et al., 1999), whereas 18-β-glycyrrhetinic acid has been demonstrated to dephosphorylate Cx43 by type 1 or type 2A protein phosphatases (Guan et al., 1996). For carbenoxolone, several authors reported reduced Cx43 expression after prolonged (6–24 hours) carbenoxolone exposure (Herrero-González et al., 2009; Wang et al., 2009; Yulyana et al., 2013; Kim et al., 2017). Goldberg et al. (1996) reported no changes in connexin expression or location but alterations of HC packing in GJ plaques (Goldberg et al., 1996). Carbenoxolone is not a specific connexin channel blocker; for example, it is a commonly used drug in the treatment of gastrointestinal ulceration in Asian countries. Because of its steroid hormone backbone structure, it has intrinsic mineralocorticoid effects. Additionally, it has several other side effects (reviewed in Connors, 2012): it inhibits 11-β-hydroxysteroid dehydrogenase and thereby influences glucocorticoid synthesis (Monder et al., 1989; Sandeep et al., 2004), inhibits voltage-gated Ca^{2+} currents [IC_{50} ~50 μM (Vessey et al., 2004)], has direct effects on GABA receptors (Connors, 2012), inhibits synaptic currents and action potentials (Rekling et al., 2000; Rouach et al., 2003; Tovar et al., 2009; Beaumont and Maccaferri, 2011), and acts as an anti-inflammatory agent (Amagaya et al., 1984; Inoue et al., 1989), which may all contribute to the observed effects beyond the effect on connexin channels as illustrated in, e.g., cardiac ischemia models (Haleagrahara et al., 2011). Carbenoxolone also inhibits pannexin channels with an IC_{50} ~5 μM (Bruzzone et al., 2005), whereas higher concentrations (~50 μM or higher) are necessary to inhibit GJs (see Table 1 in Spray et al., 2006); of note, connexin HCs are also inhibited by concentrations in the IC_{50} range of 5 μM. Also $P2X_7$ receptors are inhibited by carbenoxolone with an IC_{50} in the range of 175 nM (Suadicani et al., 2006).

Other nonspecific connexin channel inhibitors include long-chain alcohols such as heptanol and octanol, which incorporate in the plasma membrane and act by altering membrane fluidity that somehow affects the channel (Bastiaanse et al., 1993). Another lipophilic agent is halothane, an inhalational general anesthetic that inhibits GJs at supra-anesthetic concentrations (Wentlandt et al., 2006)) in the order of 2 mM (Burt and Spray, 1989); it inhibits GJs by decreasing the open probability (He and Burt, 2000). Fatty acids like oleic acid, a mono-unsaturated fatty acid present in olive oil with various biologic effects, inhibits GJs indirectly via PKC phosphorylation, as mentioned above. Fatty acid amides like anandamide, an endogenous cannabinoid receptor agonist, also blocks GJs (Venance et al., 1995). The fatty acid arachidonic acid, a poly-unsaturated fatty acid in plasma membrane phospholipids involved in inflammatory signaling, takes a special place, because it inhibits GJs but promotes HC opening (Contreras et al., 2002; De Vuyst et al., 2007,2009). Its K_D for GJ inhibition is ~4 μM, and inhibition has been linked to decreased open probability (Schmilinsky-Fluri et al., 1997).

Fenamates like flufenamic acid, niflumic acid, and meclofenamic acid are nonsteroidal anti-inflammatory molecules that inhibit cyclo-oxygenase. They inhibit GJs with an IC_{50} in the order of 25–40 μM (Harks et al., 2001; Srinivas and Spray, 2003) and have been also demonstrated to block HCs (Gomes et al., 2005). Flufenamic acid acts by decreasing the open probability (Srinivas and Spray, 2003). Quinine and mefloquine are antimalarial drugs that display some specificity for inhibiting Cx36 and Cx50; mefloquine displays an IC_{50} of 300 nM and ~1 μM for Cx36 and Cx50 (Cruikshank et al., 2004), i.e., very low concentrations compared with other connexin channel inhibitors, making it the most potent GJ inhibitor currently known. However, the downside is that the substance also has multiple side effects (see Verselis and Srinivas, 2013). Quinine has been shown to promote HC opening (Stout et al., 2002), similar to arachidonic acid. Derivatives of the triarylmethane cotrimoxazole, an antibiotic, also has connexin specificity targeted to Cx50; it acts with an IC_{50} of 1–2 μM and has a 10-fold higher specificity for Cx50 compared with other connexins as well as Na^+ and K^+ channels (Bodendiek et al., 2012; reviewed in Verselis and Srinivas, 2013).

Several other connexin channel-inhibiting compounds are best known for their other actions, including 2-amino ethoxydiphenyl borate, a blocker of IP_3 receptors and store-operated Ca^{2+} entry; polyamines like

TABLE 2
Overview of effects of agents acting at various levels of the connexin life cycle

Agent	Expression	HC Assembly	Trafficking	HC Docking	Localization (ID)	GJ Coupling	Degradation	References
Intracellular H^+, Na^+, Ca^{2+}						↓		Burt (1987), Maurer and Weingart (1987), Noma and Tsuboi (1987)
Monensin (ionophore)		↓						Puranam et al. (1993)
Brefeldin A (ER to Golgi protein transport inhibitor)			↓					Musil and Goodenough (1995)
Lactacystin, ALLN (proteasomal inhibitors							↓ (protea-some)	Laing et al. (1998)
Leupeptin (inhibitor of cathepsin B, H, and L)							↓ (lyso-some)	Leithe and Rivedal (2004)
PKA activation (cAMP, forskolin, IBMX)			↑			↑		De Mello (1984), Burt and Spray (1989), Kwak and Jongsma (1996)
PKC activation	↑↓*					↑*	↑*	Kwak et al. (1995a), De Mello (1997), Polontchouk et al. (2002), Lin et al. (2003a)
Staurosporine/PKC inhibition						↓*		Sáez et al. (1997)
Norepinephrine (α-adrenoceptors)	↑					↓ acute[#] ↑ chronic		De Mello (1997), Salameh et al. (2006), Rojas Gomez et al. (2008)
Isoprenaline (β-adrenoceptors)	↑					↑		Kwak and Jongsma, (1996), Salameh et al. (2006)
Endothelin, angiotensin	↑					↑ chronic		De Mello and Altieri (1992), Polontchouk et al. (2002)
Nicotine	↓							Tsai et al. (2004), Haussig et al. (2008), Duerrschmidt et al. (2012)
Triiodothyronine	↑							Stock and Sies (2000)
FSH	↑							Kalma et al. (2004)
VEGF, bFGF	↑					↓		Doble and Kardami (1995), Doble et al. (2000), Suarez and Ballmer-Hofer (2001), Pimentel et al. (2002)
TNF-α	↑↓[§]							van Rijen et al. (1998), Eugenín et al. (2001), Salameh et al. (2004)
Isoflurane, halothane						↓		Burt and Spray (1989)
Octanol, heptanol, palmitoleic acid						↓		Delmar et al. (1987), Burt et al. (1991), Dhein et al. (1999)
carbenoxolone						↓		Samoilova et al. (2003)
Gap26, Gap27				↓				Chaytor et al. (1997)
AAP10, rotigaptide	↑				↑	↑		Dhein et al. (1994), Müller et al. (1997b), Weng et al. (2002), Xing et al. (2003), Easton et al. (2009)
Lycopene (carotenoid from tomato)	↑							Livny et al. (2002), Zhang et al. (2016b)
TCDD (tumor promoting agent)	↓							Baker et al. (1995)

AAP10, antiarrhythmic peptide 10 (H-Gly-Ala-Gly-Hyp-Pro-Tyr-CONH2); ALLN, acetyl-leucyl-leucyl-norleucinal; bFGF, basic fibroblast growth factor; FSH, follicle stimulating hormone; Gap26, Gap27, peptide analogs to extracellular loops (see Fig. 1); HC, hemichannel; ID, intercalated disk; PKA, protein kinase A; PKC, protein kinase C; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TNFα, tumor necrosis factor α; VEGF, vascular endothelial growth factor.

*depends on PKC isoform; [#]probably indirect effect via $[Ca^{2+}]_i$; [§]both effects have been described.

spermine and spermidine acting on NMDA channels; and several other targets, 5-nitro-2-(3-phenylpropylamino)benzoic acid, a chloride channel blocker; disodium 4,4′-diisothio cyanatostilbene-2,2′-disulfonate, an anion transport blocker; and certain triphenylmethanes, triphenylethanes, triarylmethanes, and cyclodextrins (Bodendiek and Raman, 2010).

B. Peptide Modulators of Connexin Channels

Improved specificity has been sought by making use of peptides that interact, directly or indirectly, with the connexin protein and thereby modulate, inhibit, or promote its channel function. Most of the peptides used are identical to a sequence on the connexin protein, e.g., Gap26 and Gap27, whereas others like AAP10 are unrelated to the connexin protein.

1. Mimetic Peptides of the Extracellular Loops.

a. GAP26 and GAP27 peptides. Warner et al. (1995) were the first to report that peptides identical to sequences on the connexin EL domains (called "connexin mimetic peptides") inhibited the synchrony of chick myoball contractions used as an indirect assay of GJ coupling (Warner et al., 1995). The starting point for this work was the hypothesis that supplying exogenous peptides identical to crucial domains on the ELs would interact with the ELs and thereby interfere with or prevent the docking of opposed HCs. By using their myoball approach, they identified several conserved motifs in the ELs that inhibited GJs when applied as synthetic peptides. For EL1, the crucial conserved connexin motifs were QPG and SHVR, whereas the conserved SRPTEK motif was crucial for EL2 (Warner et al., 1995). Peptides containing these motifs were, respectively, dubbed Gap26 (EL1) and Gap27 (EL2). For Gap26, the crucial motifs rather look like VCYD and FPISH for various connexins (e.g., Cx26, Cx32, Cx37, Cx40, and Cx43 and others) instead of QPG and SHVR (VCYD and FPISH motifs are illustrated in Fig. 1). The ability of SRPTEK-containing peptides to inhibit GJ coupling was enhanced by adding several amino acids from the putative membrane-spanning region. For example, Gap27 (Cx43 sequence SRPTEKTIFII in human and mouse) contains three amino acids that dig into the plasma membrane at TM4 (Fig. 1). Inhibition of GJs by Gap26/Gap27 is characterized by an IC_{50} of 20–30 μM (Chaytor et al., 1997) and the peptides need to be applied at 200–300 μM to obtain maximal effects. However, these data were not obtained directly by measuring GJ coupling but by indirect measurements consisting of phenylephrine-induced rhythmic activity of endothelium-denuded rabbit superior mesenteric artery rings. GJ block by Gap26/27 is never complete [~95% complete for Gap26 (Desplantez et al., 2012b)]. Although Gap26 and Gap27 contain the conserved sequences VCYDXXFPISH and SRPTEK, respectively, they also contain amino acids that are less conserved. For example, the Gap27 sequence for Cx43 is SRPTEKTIFII (see Fig. 1; further referred to as ^{43}Gap27), which is the same sequence as for Cx37 (hence its denomination as ^{37,43}Gap27) but slightly different for Cx40: SRPTEKNVFIV (^{40}Gap27). By contrast, the Gap26 sequence for Cx37 and Cx40 is the same: VCYDQAFPISHIR (^{37,40}Gap26) but different for Cx43 (VCYDKSFPISHVR, Fig. 1). Experimental work with these peptides (or close derivatives) on vascular cells has demonstrated distinctive effects on Cx37, Cx40, and Cx43 (Kwak and Jongsma, 1999; Ujiie et al., 2003; Martin et al., 2005; Young et al., 2008; Billaud et al., 2011), suggesting they display some isoform, specificity.

The exact way of how Gap26 and Gap27 inhibit GJs is currently not known but several hypotheses exist (Evans and Boitano, 2001). First, the peptides may interact with the ELs of HCs, thereby preventing the interaction and docking of two complementary HCs to form a new GJ channel (Leybaert et al., 2003). As a result, fewer HCs will be incorporated into GJs (see *section II.B*). Because the connexin half-life is 1–5 hours, GJ inhibition would in this case be expected to occur on a similar time scale as hindered docking would decrease the number of GJ channels as a result of the ongoing GJ disassembly process. The kinetics of Gap26/Gap27 inhibition of Cx43-based GJs has an estimated half-time of ~13 minutes based on electrophysiological measurements (Desplantez et al., 2012b). Indirect measurements based on Ca^{2+} waves indicate a half-time in the order of 15–30 minutes for Gap26 and 30–60 minutes for Gap27 (Boitano and Evans, 2000). By contrast, exposure times as long as 6 hours have been reported to have no effect on FRAP-based dye coupling (Decrock et al., 2009a) and 24 hours is necessary to see an effect on coupling (De Bock et al., 2011). At the other extreme are observations based on capacitance measurements that indicate GJ inhibition within 5 minutes (Matchkov et al., 2006). Possibly, capacitance measurements may be influenced by effects on HCs, because those channels are typically blocked on a time scale of a few minutes (Desplantez et al., 2012b; Wang et al., 2012a) (HC effects are discussed below). It is currently not known how Gap26/27 interact with the ELs; there are no reports on direct molecular interactions based on surface plasmon resonance or NMR studies. Work with atomic force microscopy whereby Gap26 was covalently linked to the scanning tip has demonstrated interaction with the Cx43 ELs based on the unbinding force experienced by retracting the scanning tip after interaction was established (Liu et al., 2006). As pointed out earlier (see *section II.B.2*), HC docking involves EL1-EL1 and EL2-EL2 interactions between opposed connexins, but EL1-EL2 and EL2-EL1 interactions are also possible. Recently, the Green group performed competition experiments with Peptide5, a connexin HC-inhibiting peptide based on the EL2 SRPTEK ("Gap27") motif (Fig. 1), with various neighboring

EL2-based sequences (Kim et al., 2017). They found that Peptide5 inhibition was counteracted by equimolar addition of an EL2 peptide with a sequence N-terminally adjacent to the Peptide5 sequence, indicating that Peptide5 interacts with EL2. Most interestingly, a peptide composed of the entire EL1 sequence acted synergistically with Peptide5, giving significantly increased inhibition. Thus, EL2-derived peptides appear to interact with EL2 and not with EL1; it remains to be determined whether EL1-based peptides interact with EL1. Additionally, combination of EL1- and EL2-based peptides may provide stronger inhibition.

A second possibility for Gap26/27 effects may relate to direct interactions of these peptides with existing GJs, resulting either in the separation of HCs from existing GJs or in effects on GJ gating. Separation of docked HCs is in principle possible, and continuation of this process would result in unzippering of the GJ followed by internalization and breakdown. Solan and Lampe (2014) have suggested that the switch between unzippering and uptake of the complete GJ as an annular junction (see *section II.B.4*) is determined by distinct Cx43 phosphorylation patterns (Solan and Lampe, 2014). Although it is conceivable that Gap peptides may have access to the intercellular space and HC docking region via the edge of the junctional plaque, there is currently very little evidence for the unzippering scenario given the tenacity of adhesion of docked HCs. The other possibility is that Gap26/27 interaction with GJs results in effects on GJ channel gating properties. Such effect has been described for an EL2 peptide called P180-195 composed of amino acids Ser-180–Gln-195 of Cx43 (Fig. 1), which appeared to decrease the dwelling in the subconductance (residual) state without pronounced alterations to the main conductance state (Kwak and Jongsma, 1999). However, peptide exposure times were very long (overnight) in this study. In the absence of any additional evidence, the possibility of direct actions of the Gap peptides on the gating or unzippering of GJs remains open for further investigations.

Thus far, most evidence points to interaction of Gap26/27 peptides with HCs not incorporated in GJs (see Fig. 3 in Leybaert et al., 2003). Most notably, Gap26/27 peptides inhibit HCs faster than GJs: macroscopic current measurements have suggested HC inhibition within 5 minutes (Desplantez et al., 2012b) and analysis at the unitary current level has demonstrated a half-time of ~100 seconds (time constant τ of 148 seconds) and ~150 seconds (τ = 223 seconds) for Gap26 and Gap27, respectively (Wang et al., 2012a). Removal of inhibition occurs within a time frame of 2.6 minutes (Desplantez et al., 2012b). As discussed earlier, GJ inhibition takes tens of minutes to hours, indicating that the peptides first interact with HCs and subsequently prevent the docking process, thereby inhibiting GJs. Although HC block occurs within minutes, this is still slow in terms of action dynamics on a channel protein. One reason could be that the interaction site on the ELs is not immediately accessible and needs some molecular rearrangements or unfolding. Another possibility is that there are other interactions with connexin domains that are buried deeper into the channel pore or that are only accessible when the channel is open; the latter option still needs to be tested by evaluating the use dependence of block. In fact, the time-dependence curve of HC block in Wang et al. (2012a) may contain some use-dependent effect, because the protocol of this experiment involved repetitive application of HC opening voltage steps. In terms of mechanisms of HC inhibition, the classic possibilities are decreased number of channels, pore block, decreased unitary conductance, or effects on gating (reviewed in Wang et al., 2013a). Effects on the number of channels and single channel conductance have been excluded (Wang et al., 2012a), at least for an exposure time of 30 minutes. Pore block occurs at high concentrations, in the order of 1 mM, in which case control peptides (e.g., with a scrambled sequence) as well as active peptide inhibit HCs (Wang et al., 2012a), demonstrating that block becomes unspecific at these high concentrations. The most probable effect of Gap26/27 resides at the level of channel gating: Wang et al. (2012a) reported that Gap26/27 decreases the dwelling in the main conductance state without affecting the subconductance state (Wang et al., 2013a); these findings are opposite of the effects reported by Kwak and Jongsma (1999) with the P180-195 EL2 peptide (Fig. 1), which demonstrated decreased dwelling in the subconductance state without changes for the main conductance state. As transitions from fully closed to the main open state involve the slow loop gate, it can be concluded that Gap26/27 inhibition results from closure of the loop gate. Gap26/27 also have other effects on gating, in that they increase the V_m threshold for voltage activation. Although substantial mechanistic insights have been obtained on mechanisms of Gap26/27 inhibition of electrically triggered HC opening, little experimental evidence is available on their effect on gating mechanisms involved in chemically triggered HC opening. However, as the slow loop gate plays a central role in chemical gating, it looks obvious to suppose that Gap26/27 would also inhibit loop gate opening triggered by, e.g., $[Ca^{2+}]_i$ elevation or exposure to proinflammatory or ischemic conditions. Just like is the case for GJs, Gap26/27 block of HCs is incomplete. Gap26 HC block appears to be less complete (~65% inhibition) than observed with Gap27 (~84% inhibition; at supramaximal concentrations). On the other hand, the IC_{50} of Gap26 is lower (~80 μM) than for Gap27 (~160 μM) (Wang et al., 2012a). Note that the IC_{50} of these peptides for HC inhibition are higher than those reported by Chaytor et al. (1997) for inhibition of GJs; however, as

mentioned earlier, the assay in the Chaytor et al. (1997) study was a very indirect one based on rhythmicity of blood vessel rings exposed to phenylephrine, whereas the HC assays of Wang et al. (2012a) were based on measurements of unitary HC opening activities. Although Gap26/27 peptides have some specificity for different connexin isoforms, their selectivity has been challenged, because they were found also to inhibit Panx1 channels (Dahl, 2007; Wang et al., 2007a). Limited effects of Gap26/27 peptides on other proteins like Panx1 channels are not unexpected, given the high concentration of 200 μM or higher needed to block connexin channels; however, the effect of Gap26/27 on connexin channels is much stronger than on Panx1 channels. It is therefore advised to use concentrations less than 200 μM, because nonspecific effects will progressively appear; at 1 mM, nonspecific block is maximal with no difference between active and scrambled peptide sequences (Wang et al., 2012a).

b. Peptide5. Peptide5 is a peptide based on the SRPTEK domain with sequence VDCFLSRPTEKT (Fig. 1) first reported by O'Carroll et al. (2008). This peptide came out as most potent after a screen of several peptides mimicking sequences on the Cx43 ELs (including some containing TM portions) for their potential to inhibit cell swelling, astrogliosis, and neuronal cell death in an in vitro model of spinal cord injury. They found that Peptide5 inhibited HCs at concentrations of 5 μM, whereas hundreds of micromolars were necessary to inhibit GJs. Follow up work demonstrated significant neuroprotective and inflammation dampening effects of this peptide in various models including brain ischemia in fetal sheep (Davidson et al., 2012b, 2014), retinal ischemia (Danesh-Meyer et al., 2012), and spinal cord injury (O'Carroll et al., 2013) and subsequently extensively tested for its neuroprotective potential (see *section VIII*). Recent work from Kim et al. (2017) demonstrated that HC block is very sensitive to alterations of the Peptide5 sequence (either single amino acid substitutions for Ala or truncations at the NT or CT side) and that the SRPTEKT sequence was not sufficient on its own to block HCs (Kim et al., 2017). By contrast, GJ block was not significantly altered by these modifications and SRPTEKT acted equally well as Peptide5. This suggests distinct interactions sites for Peptide5 inhibition of HCs and GJs. Most interestingly, combining Peptide5 with EL1 peptide (entire loop sequence) increased the potency of HC inhibition. Given the fact that HC inhibition (but not GJ inhibition) is very sensitive to sequence alterations of Peptide5, including single amino acid substitutions outside the SRPTEKT sequence, suggests a possible sequence specificity for HC inhibition, an option that needs to be further tested. Peptide5 inhibition of GJs appeared to be mediated by altered distribution of Cx43 without influencing the expression level (Kim et al., 2017). Currently there are no data available on Peptide5 effects at single channel resolution to judge its effect on unitary conductance and gating.

2. Mimetic Peptides of Intracellular Connexin Sequences.

a. GAP19 and L2-specific hemichannel blocking peptides. Although Peptide5 inhibits HCs only at low concentrations and gives combined GJ/HC inhibition at high concentrations, Gap19 and L2 peptides inhibit HCs while they prevent the closure of GJs, i.e., they have opposite effects on GJs and HCs (Fig. 2A). Gap19 is a peptide mimicking a nine amino acid sequence on the CL of Cx43 located within the L2 sequence (Fig. 1). Combined work of the Bultynck and Leybaert groups has demonstrated that L2 peptide, which was known to prevent GJ closure upon acidification (see *section II.E.2*) unexpectedly inhibited Cx43 HCs (Ponsaerts et al., 2010; reviewed in Iyyathurai et al., 2013). They further identified Gap19 peptide as a sequence within the L2 domain that is flanked at both sides by α-helices at pH 5.8 (Duffy et al., 2002). In previous work with Gap26/27, mimetic peptides of the CL like Gap19 were used as inactive control peptides for GJ studies. In line with this, Gap19 had no acute effect on Cx43 GJs while it slightly promoted GJ coupling when applied for 24 hours or more (Wang et al., 2013c). Similar findings have been reported for L2 peptide, which decreases the frequency of transitions from the main to the residual state of GJ channels (Seki et al., 2004). Surprisingly, it was found that Gap19, like L2 peptide, inhibited Cx43 HCs (Wang et al., 2013c). They further demonstrated by surface plasmon resonance experiments that Gap19 interacted with the CT and that CT9 peptide inhibited Gap19 HC inhibition in a dose-dependent manner, indicating that Gap19 prevents CL interaction with the CT9 region. As explained previously, CT-CL interaction is necessary for Cx43 HCs to become available for opening (see *section II.E.5*). As a result, Gap19 prevention of CT-CL interaction will bring Cx43 HCs into a state where they are unavailable for opening. Interaction of Gap19 with the CT target is characterized by a K_d of ~2.5 μM. Although the CT9 domain is a target of Gap19, other CT sites may also be involved, as was observed with L2 peptide in the context of GJ coupling (see *section II.E.2*). Gap19 has some intrinsic membrane permeability but coupling of Gap19 to the TAT translocation sequence strongly increases its membrane permeability; experiments with TAT-Gap19 indicated an IC_{50} of ~7 μM, i.e., close to the K_d for its interaction with the CT. Based on loss of channel function with the Cx43 I130T mutant (Shibayama et al., 2005) (Fig. 1), a mutation associated with ODDD (see *section VII*), an I130A mutant Gap19 version was tested and found to be inactive and thus useful as a control peptide (Wang et al., 2013c). Gap19 was tested on Cx40 HCs and Panx1 channels, on which it had no effects (Wang et al., 2013c). Of note, Gap19 contains several charged residues (4 Lys and 1 Glu) on a total of nine amino acids and its interaction with the CT

may thus involve substantial electrostatic interactions. The interaction spectrum of the much longer L2 peptide (26 amino acids) will certainly be more elaborate and may ascertain specificity. L2 does not enter the cells spontaneously and needs to be coupled to a translocation motif such as TAT; mutant H126K/I130N L2 is the corresponding inactive control peptide (Ponsaerts et al., 2010). Interestingly, Gap19 has been used to investigate the role of Cx43 HCs in isoproterenol-induced cardiac arrhythmias, demonstrating a drastic improvement of isoproterenol-challenged animal mortality (González et al., 2015). Work with this peptide in mice has furthermore demonstrated it modestly reduces infarct size after myocardial ischemia/reperfusion (Wang et al., 2013c). TAT-linked Gap19 was shown to impair spatial short-term memory when injected into mice brain ventricles (Walrave et al., 2016). TAT-linked L2 peptide potently inhibited norepinephrine-induced vasoconstriction of rat small mesenteric arteries (Bol et al., 2016) and was demonstrated to inhibit fear memory consolidation when injected into rat basolateral amygdala (Stehberg et al., 2012).

Just like $[Ca^{2+}]_i$, proinflammatory cytokines and metabolic inhibition distinctly influence HCs and GJs, this also appears to be the case for Gap19 and L2 peptides. Taking into account that both peptides interfere with CT-CL interaction and are identical to the sequence of domains crucial for this interaction, this suggests that the process of docking and GJ formation induces conformational changes that switch the outcome of CT-CL interaction from necessary for HC opening to inhibition of GJ.

Interestingly, the L2 peptide has been further explored in terms of its pharmacophore for interacting with the Cx43 CT, with the RXP-E motif appearing as an interesting starting point from which other peptides and petidomimetics have been developed (see *section II.E.2*) (Shibayama et al., 2006b; Lewandowski et al., 2008; Verma et al., 2009). In particular, the linear peptide RRNYRRNY, the cyclic peptide CyRP-71, and the peptidomimetic molecule ZP2519 were demonstrated to target the Cx43 CT and to prevent Cx43-based GJ closure under low pH conditions (Verma et al., 2009,2010). These substances are of potential translational value for preventing postischemic GJ closure. Moreover, these molecules are potential HC blockers and may thus have two-sided actions directed at preventing GJ closure as well as inhibiting HC opening. Interestingly, RRNYRRNY was recently demonstrated to act as a Cx43 HC blocker, not only of plasma membrane HCs but also of HCs in subsarcolemmal mitochondria (Gadicherla et al., 2017). As a result, RRNYRRNY displays three levels of action on Cx43-based channels: prevention of GJ closure, inhibition of HCs in the plasma membrane, and inhibition of mitochondrial HCs (mitochondrial connexin channels are further discussed in *section V.A*).

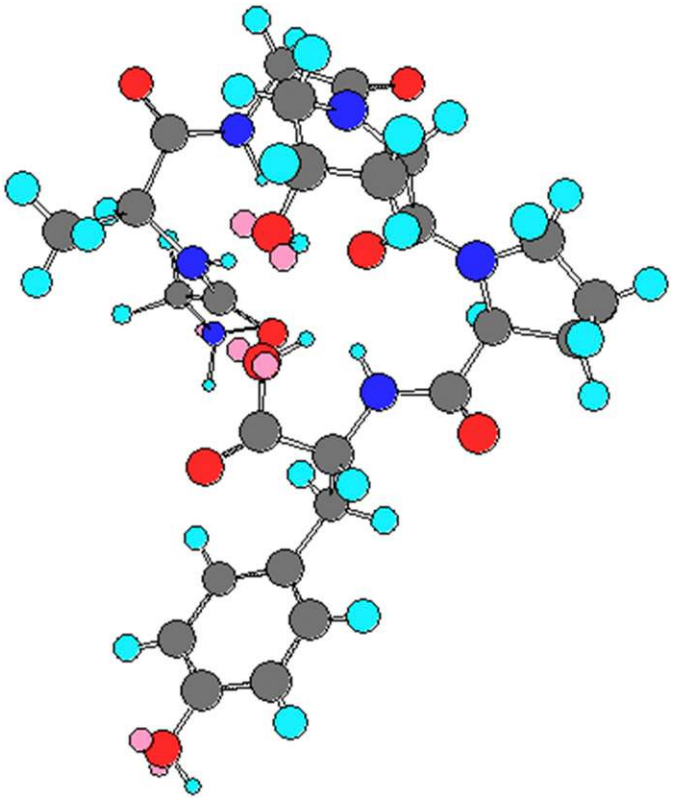

Fig. 3. Chemical structure of AAP10.

2. JM2 Peptide. Recently, the Gourdie group reported that JM2 peptide, composed of 15 amino acids in the juxtamembrane region of the Cx43 CT (see Fig. 1), inhibited ATP release triggered by low extracellular Ca^{2+} in human microvascular endothelial cells (Calder et al., 2015). The peptide overlaps with the crucial juxtamembrane (JM) domain (amino acids 234–243, indicated in Fig. 1) that functions as a microtubule binding site, which is part of a larger domain stretching from amino acid 228 to 263 (Giepmans et al., 2001). JM2 peptide was tested on hemichannel ATP release, but its effects on gap junctions were not investigated.

3. CT9 Peptide. As discussed earlier, CT9 peptide removes the high $[Ca^{2+}]_i$ brake on Cx43 HC opening (see *section II.E.5*). As a result, CT9 acts as a promoter of $[Ca^{2+}]_i$-linked HC opening (Fig. 2B), as illustrated recently in vascular smooth muscle cells (Bol et al., 2016). CT10, which contains an additional Ser (Ser-373), has the same effect (Ponsaerts et al., 2010), but CT9 is the smallest active sequence (the Ser was added as a linker to the TAT translocation motif). Moreover, CT9ΔI peptide that lacks the last CT-located Ile (Ile-382 in the Cx43 protein) and therefore does not compete with Cx43–ZO-1 interaction (see *section II.B.3*) still displays its HC activity-enhancing effect (De Bock et al., 2012). Similar to Gap19, L2, and RRNYRRNY, CT9 has its target inside the cell (L2/Gap19 sequences on the CL) and needs to be fused to a translocation sequence to improve its membrane permeation (TAT-CT9).

αCT1 is another form of CT9 peptide, which is linked to the antennapedia membrane translocation sequence instead of the TAT sequence. Interestingly, αCT1 promotes HC incorporation into GJs and increases plaque size by facilitating HC disconnection from ZO-1–linked cytoskeletal elements as a result of competition between the peptide and the endogenous Cx43 CT9 motif (Hunter et al., 2005; Palatinus et al., 2012; Rhett et al., 2013) (see *section II.B.3*). αCT1 reduces inducible arrhythmias after cryoinjury applied to the left ventricle in the heart and prevents Cx43 lateralization/remodeling; it also increases Cx43 phosphorylation at Ser-368 in a PKCε-dependent manner (O'Quinn et al., 2011). These effects are exerted not within seconds per minute characteristic for CT9 effects on HC gating but on a time scale of hours that is characteristic for the cardiac Cx43 life cycle. As a result CT9, fused to either TAT or the antennapedia sequence, has two effects: within minutes it promotes $[Ca^{2+}]_i$-linked HC opening, whereas within hours it promotes HC incorporation into GJs, thereby reducing the HC pool in the plasma membrane. If CT9 is used with the purpose of promoting HC incorporation into GJs (long-term effect to improve GJ coupling), it is advisable to consider combining its administration together with an HC blocker to prevent the potentially inappropriate acute HC-opening effect of CT9-based peptides.

4. Note on Hemichannel-Targeting Strategies. Currently Gap19, L2, and RRNYRRNY peptide (Fig. 1) are the only known molecules that inhibit Cx43 HCs without inhibiting GJs. Peptide5 is an HC blocker at low (5 μM) concentrations that blocks GJs at higher concentrations; Gap26/27 inhibit HCs within minutes, with delayed effects on GJs (hours). Two other (nonpeptide) compounds namely boldine (Hernandez-Salinas et al., 2013) and D4 (Cea et al., 2016b) have been reported to act as connexin HC blockers. Boldine is an alkaloid extracted from the boldo tree but the chemical identity of D4 was not revealed. Recently, Yi et al. (2017) demonstrated that boldine inhibited HCs but not GJs in astrocytes at concentrations of 0.1 and 0.5 mM in cultures and acute brain slices. These authors furthermore showed that long-term in vivo boldine administration (12 weeks, administered in the drinking water) inhibited HCs but not GJs; in APP/PS1 mice such treatment reduced $[Ca^{2+}]_i$ elevation in astrocytes, tempered gliotransmitter release and mitochondrial superoxide ion load, and reduced dystrophic neurite counts (Yi et al., 2017). Amyloid load was not affected and behavior/cognition were not investigated.

Certain glycyrrhetinic acid-based molecules have been claimed to act in an HC-specific manner but there is little convincing evidence to support this claimed selectivity (Takeuchi et al., 2011). Lanthanum ions (La^{3+}) block HCs and indeed do not inhibit GJs (Anselmi et al., 2008); however, this ion has manifold other effects, including the inhibition of Ca^{2+} channels (Mlinar and Enyeart, 1993; Young et al., 2002).

Besides peptides, two ODDD Cx43 mutants have been characterized to display increased HC function combined with decreased gap junctional function. These mutants are G138R and G60S (see Fig. 1) (Dobrowolski et al., 2007, 2008; Kozoriz et al., 2013). As such, these mutants are interesting tools to distinguish HC function from GJ function. The G138R mutant has been used to investigate the consequences of increased HC function in brain slice experiments (Torres et al., 2012), whereas the G60S mutant was demonstrated to increase infarct size in animal stroke models (Kozoriz et al., 2013). Another interesting mutant not linked to ODDD is R76W, which has impaired GJ function but preserved HC function (Xu et al., 2015) and G8V that has preserved GJ function but increased HC opening (Wang et al., 2015a) (Fig. 1).

3. Peptides not Mimicking Connexin Sequences: The Case of AAP10. The most striking and best characterized peptide with pronounced effects on GJ channels, which is not based on a connexin protein sequence, is AAP10 peptide and analogs. AAP10 stands for antiarrhythmic peptide 10 and is derived from a naturally occurring antiarrhythmic peptide isolated from bovine atria, which enhanced the synchronization of cultivated clusters of spontaneously beating embryonic chicken cardiomyocytes (Aonuma et al., 1980). Starting from this original antiarrhythmic peptide, a number of synthetic derivatives was developed, among which AAP10 (GAG-4Hyp-PY) (illustrated in Fig. 3) was most active and chosen as lead substance (Grover and Dhein, 2001). The hypothesis that "enhanced synchronization of cardiomyocyte clusters" may represent an increase in intercellular coupling could be verified: simulated ischemia (hypoxia combined with glucose deficit) leads to a reduction in conduction velocity in guinea pig papillary muscles that could be prevented by 1 μM AAP10 added to the bath solution (Müller et al., 1997a). Furthermore, it was found that in dual whole cell patch clamp experiments, concentrations of 10 or 50 nM AAP10 lead to enhanced electrical coupling between pairs of cardiomyocytes without affecting the sodium, calcium, potassium, or other transmembrane ionic currents (Müller et al., 1997a,b; Weng et al., 2002; Hagen et al., 2009). Similarly, enhanced coupling was also observed with rotigaptide, a peptide that is further discussed below (Jørgensen et al., 2005; Clarke et al., 2006). Further experiments showed that the positive effect of AAP10 on electrical coupling was increased if cells were partially uncoupled: cells exposed to low pH (6.5 via CO_2) exhibit reduced GJ conductance that was prevented by pretreatment with 50 nM AAP10 and, moreover, could also be reversed by treatment with AAP10 (Hagen et al., 2009). Besides electrical coupling, AAP10 also increased metabolic GJ coupling as evident from increased dye transfer (Hagen et al., 2009). The effects of AAP10 on GJs could be demonstrated in rat

(Hagen et al., 2009), guinea pig (Müller et al., 1997a,b; Weng et al., 2002), rabbit (Dhein et al., 1994; Jozwiak and Dhein, 2008), and human (Hagen et al., 2009) cardiomyocytes. Experiments using HeLa cells stably transfected with either Cx43, Cx40, or Cx45, i.e., the typical cardiac connexins, showed that AAP10 acts on GJs via effects on Cx43 or Cx45 but not via Cx40 (Easton et al., 2009; Hagen et al., 2009).

Because peptides often are biologically unstable, further research was focused on the structural chemistry of these peptides and on development of a radioligand binding assay to allow the development of nonpeptide drugs. Structure-activity relations together with molecular modeling, and two-dimensional NMR spectroscopy revealed that AAP10 has a semicyclic structure like a horseshoe, which is due to the two Pro residues (Dhein et al., 2010; Grover and Dhein, 1998, 2001). Moreover, it could be shown that [^{14}C]AAP10 binds to a membrane protein of rabbit cardiomyocyte membranes (Dhein et al., 2001) with a K_d in the order of 0.3–0.9 nM and maximum binding B_{max} in the order of ~42.5 pmol/mg (Jozwiak et al., 2012). The finding of the semicyclic structure and the identification of the essential chemical moieties (Grover and Dhein, 1998, 2001) led to the idea of replacing some of the amino acids by D-amino acid versions. To maintain certain groups at their positions, the order of these amino acids had to be reversed, which, using a retro-all-D amino acid design of the AAP10 template, led to ZP123 (YP-4Hyp-GAG) also named rotigaptide (Kjølbye et al., 2003; Xing et al., 2003). Due to the D-amino acids, its in vivo half-life is longer than that of AAP10. As AAP10, the D-amino acid analog rotigaptide enhanced GJ communication but did not bind to other transmembrane ion channels (Haugan et al., 2005). Further reduction to the pharmacophore allowed synthesis of the dipeptide danegaptide also called ZP1609 (Skyschally et al., 2013) and of the nonpeptide drug Gap134 [(2*S*,4*R*)-1-(2-aminoacetyl)-4-benzamido-pyrrolidine-2-carboxylic acid hydrochloride] (Butera et al., 2009).

Until this point, we have only considered the effects of these drugs on GJs, but the question arises whether antiarrhythmic peptides really exert antiarrhythmic effects and against which type of arrhythmia they may be effective. Early considerations using computer simulations about the relationship of intercellular coupling and arrhythmia started with the idea that reduced coupling might unmask local inhomogeneities in action potential duration (APD), resulting in a dispersion of APDs (Lesh et al., 1989; Müller and Dhein, 1993). Together with slowed conduction these local differences in refractoriness could lead to reentrant arrhythmia (for more detail see *section VI*). Based on this hypothesis, 256 electrode mapping was performed using isolated rabbit hearts, which demonstrated, in good accordance to the computer simulations, that under control conditions AAP10 significantly reduced dispersion in APD (Dhein et al., 1994) and that regional ischemia results in alterations of the spread of activation that were reduced by AAP10 (Dhein et al., 1994). In addition, ischemia-induced slowing of conduction in the ischemic border zone was antagonized by AAP10 (Jozwiak and Dhein, 2008). Accordingly, it was also shown that AAP10 could significantly decrease the incidence of ventricular tachycardia and ventricular fibrillation in acute ischemia (coronary ligation) (Ni et al., 2015; Sun et al., 2015a). Similarly, rotigaptide (ZP123) also reduced the incidence of ventricular fibrillation and ventricular tachycardia in acute coronary ischemia in various animal models (Hennan et al., 2006; Kjølbye et al., 2008; Su et al., 2015), prevented ischemia-induced conduction velocity slowing (Shiroshita-Takeshita et al., 2007) and suppressed arrhythmia in volume-pressure overload heart failure (Liu et al., 2014). Spiral wave reentry circuits became destabilized by enhancement of GJ coupling (Takemoto et al., 2012). As AAP10, rotigaptide also reduced APD dispersion (Dhein et al., 2003). Interestingly, AAP10 additionally reduced the inducibility of ventricular fibrillation in healed myocardial infarction (Ren et al., 2006). Of note, rotigaptide also reduced infarct size (Haugan et al., 2006; Hennan et al., 2006), although others did not observe this effect (Xing et al., 2003). Infarct size reduction has also been found with danegaptide (ZP1609) (Skyschally et al., 2013). Recent work demonstrated ZP1609 prevents cardiomyocyte hypercontracture after ischemia-reperfusion by acting on mitochondria (Boengler et al., 2017); this particular effect was, however, not linked to Cx43. AAP10 has furthermore been demonstrated to prevent drug-induced torsade de pointes arrhythmia and early afterdepolarizations (Quan et al., 2007, 2009; Ruan et al., 2014). In cardiac preparations uncoupled with lysophosphatidic acid, AAP10 antagonized ventricular tachycardia induced by programmed S1S2 stimulation (Zhou et al., 2011). In vivo, the inducibility of ventricular fibrillation by the plant toxin aconitine was significantly attenuated by AAP10 (Dhein et al., 2001).

In contrast, AAP10 did not affect burst stimulation-induced atrial fibrillation (Haugan et al., 2004). Similarly, rotigaptide did not prevent atrial tachyarrhythmia development in a chronic volume overload rabbit model (Haugan et al., 2006), whereas in a canine mitral regurgitation model, rotigaptide was effective in reducing atrial fibrillation inducibility (Guerra et al., 2006) as well as against atrial conduction slowing induced by metabolic stress (Haugan et al., 2006) or atrial stretch (Ueda et al., 2014). This was also found for Gap134 (Rossman et al., 2009). AAP10 could not reverse diabetes mellitus-induced conduction slowing in Zucker diabetic fatty rats (Olsen et al., 2013); this may be linked to the fact that this type of conduction slowing is probably due to enhanced fibrosis rather than resulting from functional electrophysiological changes

and may therefore be more difficult to reverse. Taken together, most researchers found evidence for antiarrhythmic effects of AAP10 and related peptides against ischemia-induced ventricular tachyarrhythmias, while efficacy in atrial fibrillation seems to depend on the model used and the type of atrial fibrillation. The efficacy of antiarrhythmic peptides seems to be highest in partially uncoupled tissue if uncoupling is induced by hypoxia, ischemia, or acidosis.

This leads to the question of the biochemical mechanisms of action of antiarrhythmic peptides. First of all, it has been shown that AAP10 and rotigaptide binds to a membrane protein with a K_d in the range of 0.1–0.9 nM [[^{14}C]AAP10 (Dhein et al., 2001; Jozwiak et al., 2012); [^{125}I]di-I-AAP10 (Jørgensen et al., 2005)]. A 200-kDa membrane protein could be isolated from cardiac tissue by affinity chromatography and cross-linking techniques (Weng et al., 2002), but its identity was not determined. Regarding the subsequent signal transduction cascade, the antagonization of the AAP10 effects by GDP-βS is in favor of the idea that G-proteins are involved in the signaling process (Weng et al., 2002). Furthermore, the peptides activate protein kinase Cα [AAP10 (Dhein et al., 2001; Weng et al., 2002); rotigaptide (Dhein et al., 2003)]. The effect of AAP10 on GJIC and on Cx43 phosphorylation could be completely inhibited by blocking PKC (Weng et al., 2002; Easton et al., 2009), indicating that PKC is critically involved in the signal transduction process. Consistently, several groups found enhanced phosphorylation of Cx43 after incubation of the cells/tissue with antiarrhythmic peptides [AAP10 (Dhein et al., 2001; Weng et al., 2002; Quan et al., 2007, 2009; Wang et al., 2007b; Easton et al., 2009; Sun et al., 2015a); rotigaptide (Kjølbye et al., 2008; Su et al., 2015)]. In contrast, others did not observe enhanced Cx43 phosphorylation with rotigaptide (Clarke et al., 2006). However, within the first 30 minutes of ischemia, Ser-306, Ser-297, and Ser-368 of Cx43 are dephosphorylated, whereas Ser-330 is phosphorylated. The dephosphorylation of Ser-297 and Ser-368 can be prevented by rotigaptide (Axelsen et al., 2006). Cx43 synthesis was not affected by rotigaptide (Liu et al., 2014). In contrast, AAP10 led to an increase in Cx43 mRNA expression (Easton et al., 2009).

More detailed analysis revealed that the effects of AAPs on Cx43 phosphorylation were enhanced in ischemic tissue and attained a maximum in the ischemic center while less pronounced in non-ischemic tissue (Jozwiak and Dhein, 2008), which may explain the controversial findings cited above. Accordingly, others found that the binding site density for AAP10 was enhanced during metabolic stress (Jørgensen et al., 2005). Thus, one might conclude from these finding that AAPs prevent from dephosphorylation rather than induce active phosphorylation.

Phosphorylation of Cx43 CT can alter the single channel conductance (Takens-Kwak and Jongsma, 1992; Moreno et al., 1994; Kwak et al., 1995a; Kwak and Jongsma, 1996), but also is critically involved in controlling the transfer and insertion of Cx43 into the membrane (involving the tubulin apparatus) and in the removal of Cx43 from the membrane (Lampe and Lau, 2000; Solan and Lampe, 2007; Saidi Brikci-Nigassa et al., 2012). Accordingly, two groups found that Cx43 density in the membranes is reduced in ischemia and can be preserved by AAPs (Jozwiak and Dhein, 2008; Sun et al., 2015a). Moreover, it was shown by these authors that the reduction of Cx43 and its inhibition by AAP10 was highest at the cell poles. Interestingly, AAP10 led to a higher Cx43 density at points of cell-cell contact (Easton et al., 2009), which would be in line with the assumption that the incorporation into the membrane might be enhanced. The mechanism of action of the AAPs is summarized in Fig. 4.

Regarding clinical safety, rotigaptide was evaluated in a phase I study in 200 healthy subjects and found to be safe (Kjølbye et al., 2007). Taken together, AAPs have been demonstrated to possess a pharmacological potential for the treatment of ischemia- or hypoxia-linked arrhythmias related to cellular uncoupling via Cx43 or Cx45. On the background of the observations that GJs also control growth and differentiation of cells, this approach may also be worth investigating in the area of cancer (Salameh and Dhein, 2005).

IV. Connexins in Vascular Disease

Atherosclerosis, a progressive inflammatory disease of large and medium-sized arteries, is the number one killer worldwide. The main complications of atherosclerosis, namely ischemic heart disease and stroke, are the world's first and third leading causes of death, representing 28.5% of all-cause mortality (GBD 2013 Mortality and Causes of Death, 2015). The disease involves the formation of plaques in the intima of arteries that are characterized by a dysfunctional endothelium, leukocyte and lipid accumulation, cell death, and fibrosis (Fig. 5). Atherosclerotic plaques develop predominantly at arterial locations where regular (high) laminar blood flow is disturbed, i.e., arterial bifurcations and branch points (Kwak et al., 2014). The most severe clinical events follow the rupture of a plaque (Fig. 6), which exposes prothrombotic material inside the plaque to the blood and causes sudden thrombotic occlusion of the artery at the site of disruption (Hansson et al., 2015). Various connexins have been shown to be involved in the initiation and progression of atherosclerosis. Furthermore, these proteins may also influence thrombus formation and stabilization.

Treatment of acute coronary atherothrombosis consists of procedures that allow the rapid return of blood flow to the ischemic zone of the myocardium to rescue

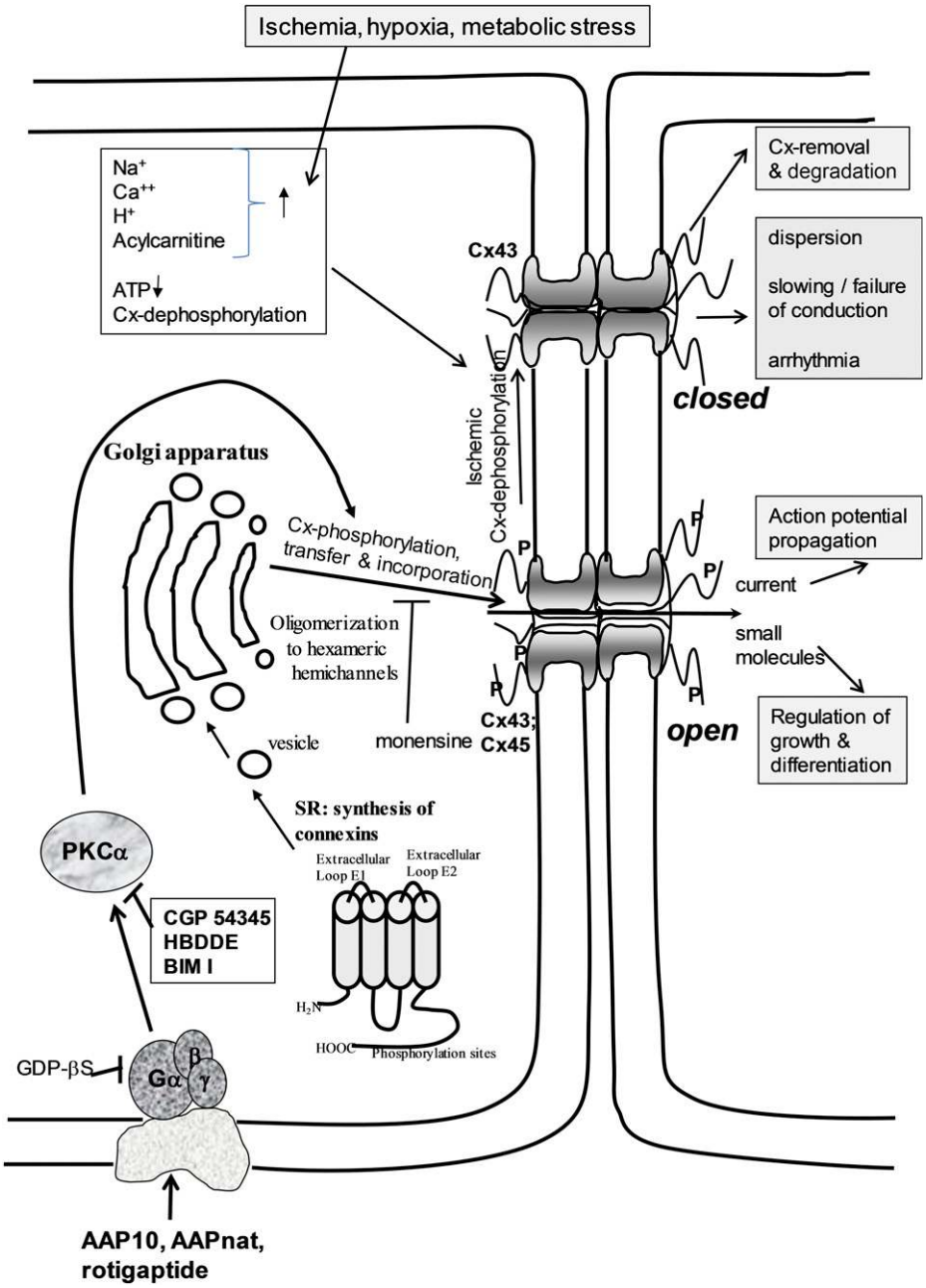

Fig. 4. Synthesis, transport, and membrane incorporation of cardiac connexins and mechanisms of ischemic damage leading to GJ closure, connexin dephosphorylation, and connexin-removal from the membrane. Molecular mechanisms and effects of antiarrhythmic peptides on GJ channels formed by Cx43 or Cx45 are also illustrated. GDP-βS, guanosine-5′-[β-thio]diphosphate trilithium salt (nonhydrolyzable GDP analog); PKCα, protein kinase Cα; CGP 54345, PKC inhibitor (inhibits only PKCα); HBDDE 2,2′,3,3′,4,4-hexahydroxy-1′-biphenyl-1-6,6′-dimethanoldimethylether, inhibitor of PKCα and PKCγ; BIM I, bisindolyl-maleimide I, inhibitor of PKCα; βI, βII, γ, δ, and ε; SR, sarcoplasmic reticulum; AAPnat, natural antiarrhythmic peptide (H-Gly-Pro-Hyp-Gly-Ala-Gly). Modified from Dhein et al. (2010).

heart muscle. However, reperfusion may paradoxically lead to further complications involving acceleration of cardiac cell death, diminished contractile function, and arrhythmias. The involvement of cardiomyocyte connexins in reperfusion injury will be described in *section IV.C*; here we will summarize recent evidence indicating an involvement of connexins in the inappropriate inflammatory response in the microcirculation that may be at the basis of ischemia-reperfusion injury (Bulluck et al., 2016).

Restenosis is the pathophysiological process involving inflammatory activation and intimal smooth muscle cell accumulation that occurs in about 10% of patients submitted to revascularization procedures of coronary, carotid, and peripheral arteries. It is in fact an excessive healing reaction of the vascular wall subjected to balloon angioplasty and endovascular stent implantation (Chaabane et al., 2013). Drug-eluting stents and the more recent drug-eluting balloons have significantly reduced but not eliminated the incidence of restenosis.

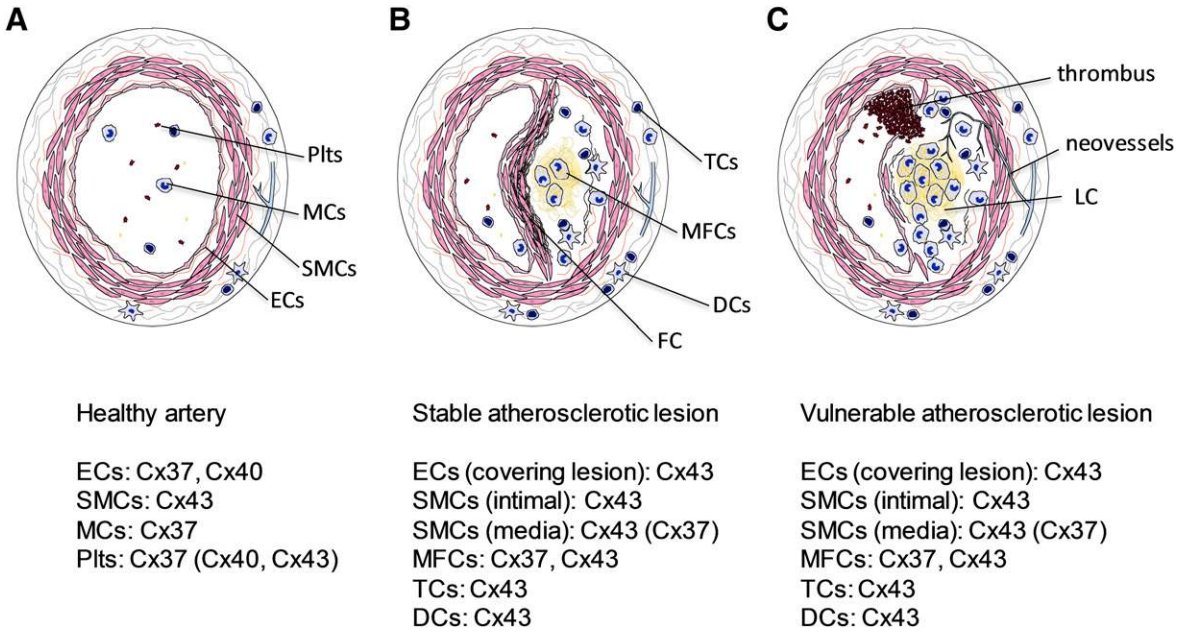

Fig. 5. Connexin expression in healthy arteries (A), stable atherosclerotic plaques with a thick fibrous cap (B), and after rupture of vulnerable lesions. Connexin expression is represented according to cell type. DCs, dendritic cells; ECs, endothelial cells; FC, fibrous cap; LC, lipid core MCs, monocytes; MFCs, macrophage foam cells; Plts, platelets; SMCs, smooth muscle cells; TC, T cells.

The role of connexins in this clinically still relevant problem will be discussed.

A. Connexins in Atherosclerosis

There is growing evidence that connexins participate in the development of atherosclerotic disease. Early support for this hypothesis came from studies analyzing atherosclerotic lesions at different disease stages in specimens of human, rabbit, or mouse origin. First, Cx43 is generally absent in the endothelium of large arteries, but it has been found in endothelial cells at the shoulder region of advanced atherosclerotic plaques (Fig. 5) (Kwak et al., 2002). Although endothelial Cx43 expression may be induced by cytokines like TGF-β that are present in excess in an atherosclerotic environment (Larson et al., 2001), Cx43 expression may already have been upregulated at this specific arterial location before the atherosclerotic lesion formed. Support for the latter premise comes from the fact that abundant Cx43 expression has been observed in rat aortic endothelial cells localized at the downstream edge of the ostia of branching vessels and at flow dividers, regions known to experience disturbed blood flow (Gabriels and Paul, 1998). Subsequently, a causal relation between the induction of endothelial Cx43 and disturbed (or oscillatory) flow has been established in various in vitro studies (DePaola et al., 1999; Kwak et al., 2005; Feaver et al., 2008). Secondly, a high level of Cx43 expression has been found in intimal macrophages and smooth muscle cells of young atheroma, whereas Cx43 expression is downregulated in intimal smooth muscle cells of more advanced lesions (Fig. 5) (Polacek et al., 1993, 1997; Blackburn et al., 1995; Kwak et al., 2002). Interestingly, the oxidized phospholipid derivative POVPC reduces Cx43 levels of vascular smooth muscle cells, enhances its phosphorylation at Ser-279/282, and increases smooth muscle cell proliferation both in vitro and in an atherosclerotic mouse model in vivo (Johnstone et al., 2009). Finally, Cx37 and Cx40 levels are also modified during the course of atherosclerotic disease in human and mice (Kwak et al., 2002). Thus, Cx40 and Cx37 disappear from endothelial cells covering advanced atherosclerotic lesions, and Cx37 expression is enhanced in macrophage foam cells (Fig. 5). Moreover, long-term hyperlipidemia (a well-known atherogenic condition) reduces endothelial Cx37 and Cx40 expression in mouse aorta, an effect that could be reversed only for Cx37 by 1 week of treatment with simvastatin, a cholesterol-lowering drug (Yeh et al., 2003). In addition, endothelial Cx37 is downregulated in response to disturbed flow (Pfenniger et al., 2012b). Altogether, these studies brought the idea that connexin expression or posttranslational modifications in connexins might evolve in atherosclerotic plaques over time, depending on the stage of the lesion and might thus affect atherogenesis.

As germ-line loss of Cx43 is lethal (Reaume et al., 1995), the role of Cx43 in atherosclerosis was first studied in $Cx43^{+/-}$ mice crossed with atherosclerosis-susceptible low-density lipoprotein (LDL) receptor-deficient ($Ldlr^{-/-}$) mice fed a high-cholesterol diet. Initial studies on $Cx43^{+/-}Ldlr^{-/-}$ mice revealed that Cx43 has an overall atherogenic effect, and that reducing Cx43 might be beneficial by both reducing plaque burden as well as stabilizing the lesions (Kwak et al., 2003). However, the exact scenario by which

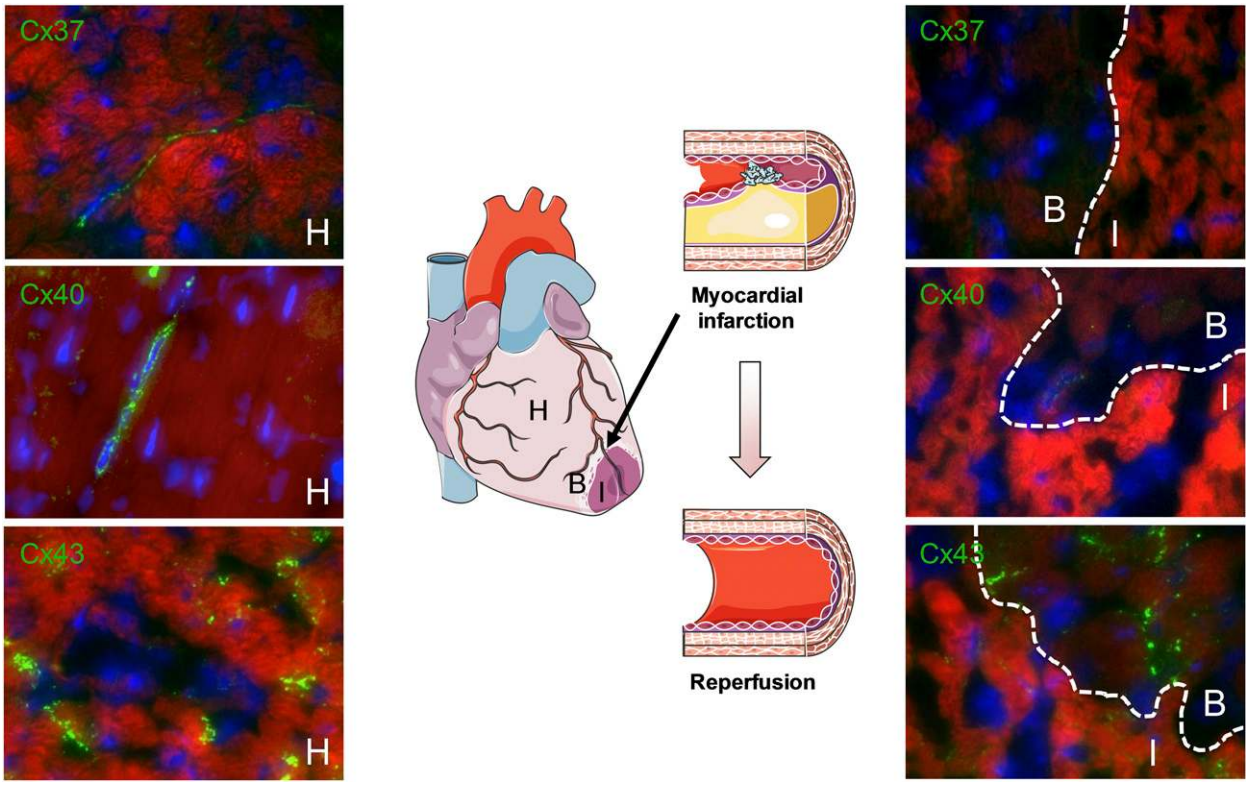

Fig. 6. Connexin expression after myocardial ischemia (due to coronary occlusion) and reperfusion in wild-type mice. (Left) Cx37, Cx40, and Cx43 expression (in green) in unaffected healthy (H) myocardium. (Right) Cx37, Cx40, and Cx43 expression (in green) at the border zone (B) of the infarcted area (I). Tissue is counterstained with Evans Blue (in red), and nuclei are stained with DAPI (in blue).

ubiquitous reduction in Cx43 leads to this dual benefit remained uncertain due to Cx43 expression in multiple atheroma-associated cell types (Wong et al., 2003). To examine specifically the role of Cx43 in immune cells, $Ldlr^{-/-}$ mice were lethally irradiated and reconstituted with $Cx43^{+/+}$, $Cx43^{+/-}$, or $Cx43^{-/-}$ hematopoietic fetal liver cells (Morel et al., 2014b). Intriguingly, the progression of atherosclerosis was lower in $Cx43^{+/-}$ chimeras compared with $Cx43^{+/+}$ and $Cx43^{-/-}$ chimeras, and their plaques contained fewer neutrophils. It turned out that chemoattraction of neutrophils, which themselves do not express Cx43, was reduced in response to supernatant secreted by $Cx43^{+/-}$ macrophages in comparison with the ones of $Cx43^{+/+}$ and $Cx43^{-/-}$ macrophages. Thus, titration of Cx43 levels in macrophages might regulate their chemoattractant secretion, leading to a reduction in atherosclerosis (Morel et al., 2014b).

In contrast to Cx43, Cx40 protects against atherosclerosis in mice by synchronizing endothelial CD73-dependent anti-inflammatory signaling, thus inhibiting leukocyte recruitment to the atherosclerotic lesion (Chadjichristos et al., 2010). Furthermore, loss of Cx37 promotes the development of atherosclerosis in apolipoprotein E-deficient ($Apoe^{-/-}$) mice. Mechanistically, it appeared that ATP release through Cx37 HCs in monocytes control the initiation of atherosclerotic plaque development by regulating their adhesion (Wong et al., 2006). Recent in vitro studies show a downregulation of Cx37 expression, inhibition of ATP release, and augmented adhesion of the human monocytic cell line THP-1 by oxidized LDL (Liu et al., 2016). These harmful effects of oxidized LDL could be in part prevented by rutaecarpine, an active component of a Chinese herbal medication (Liu et al., 2016). Finally, Cx37 deletion in apolipoprotein $E^{-/-}$ ($Apoe^{-/-}$) mice not only controls the initiation of atherosclerotic plaque formation but also increases the size of advanced lesions and abrogates the development of a stable plaque phenotype in regions exposed to oscillatory shear stress (Pfenniger et al., 2015), suggesting that local hemodynamic factors may modify the risk for Cx37-related adverse disease outcomes. A recent study examining the effects of lentiviral Cx37 interference on established abdominal aortic plaques in pigs by intravascular ultrasound revealed a reduction in plaque volume in the 8 months after lentiviral transduction, illustrating that not only local hemodynamic factors but also the disease stage itself may influence the outcome of reducing Cx37 on atherosclerosis burden (Guo et al., 2015). Although these animal studies have revealed important and diverse contribution of vascular connexins to atherogenesis, more work is needed to uncover the roles of these proteins in human disease.

During the past 15 years, a single nucleotide polymorphism (SNP) in the human *Cx37* gene (*GJA4*) has

been associated in a variety of populations with increased risk for various clinical manifestations of atherosclerosis, such as coronary artery disease, myocardial infarction, and ischemic stroke; however, these studies remained controversial as to which allele carried the risk (see for a review Meens et al., 2012). Consequently, data of multiple studies were extracted by two independent reviewers and a total of 3498 myocardial infarction cases and 3986 controls, as well as 1808 coronary artery disease cases and 1197 controls have been enrolled in a recent meta-analysis (Wen et al., 2014). This meta-analysis demonstrated that the *GJA4-1019T* allele is a risk factor for myocardial infarction and a protective factor for coronary artery disease. The *GJA4 1019C>T* SNP results in a nonconservative Pro to Ser substitution in the CT of Cx37, which has a significant impact on channel function under basal and phosphorylating conditions (Derouette et al., 2009; Morel et al., 2010; Pfenniger et al., 2010). In accordance with the above described studies using Cx37-deficient monocytes, monocytic cells expressing Cx37-319P, encoded by *GJA4-1019C*, were markedly less adhesive than cells expressing Cx37-319S, encoded by *GJA4-1019T* (Wong et al., 2006), thus suggesting that Cx37-319P polymorphic HCs may function as a protective genetic variant for plaque rupture, leading to myocardial infarction by specifically retarding recruitment of monocytes to human atherosclerotic lesions. In contrast to *GJA4 1019C>T*, SNPs located in the promoter region (*−1930 C>T*) or 3′-untranslated region (*1297 I>D*) of *GJA4*, which are presumed to affect Cx37 transcription level or mRNA stability, were not associated with altered risk for coronary artery disease (Han et al., 2008). Moreover, two SNPs in the promoter region of the *GJA5* gene, *−44G>A* and *+71A>G*, that were found to significantly reduce Cx40 transcription (Firouzi et al., 2006) could also not be associated with an altered risk for coronary artery disease or acute myocardial infarction (Pfenniger et al., 2012a; Seifi et al., 2013). Although the latter studies remain to be confirmed in large cohorts or by meta-analyses, they stir up the idea that SNPs affecting connexin channel function may be of greater importance for cardiovascular disease than SNPs affecting connexin expression levels.

B. Connexins in Thrombosis

Cx37 was the first connexin found in platelets in 2011 (Angelillo-Scherrer et al., 2011), and the additional expression of a number of other connexins [Cx43, Cx40, and Cx32 (Vaiyapuri et al., 2012)] and Panx1 (Taylor et al., 2014; Molica et al., 2015) have been reported since. Transmission electron microscopy has convincingly revealed GJ-like structures between platelets (Vaiyapuri et al., 2012)). Moreover, platelets were shown to display functional Cx37 GJ channels during the aggregation response (Angelillo-Scherrer et al., 2011). Deletion of Cx37 in mice reduced tail bleeding time, shortened the time to occlusive arterial thrombosis, accelerated mortality in a model of thromboembolism, and enhanced platelet aggregation in response to modest concentrations of the agonists ADP, thrombin, and collagen (Angelillo-Scherrer et al., 2011). Furthermore, promotion of platelet aggregation in vitro was also observed with Cx37 mimetic blocking peptides. Given the biophysical properties of Cx37 GJ channels, it was hypothesized that these channels synchronize responses in platelets brought in contact during activation by transmitting cAMP to neighboring platelets, thereby functionally inhibiting freshly recruited platelets and limiting further thrombus growth (Angelillo-Scherrer et al., 2011). Interestingly, other studies report a loss of function in $Cx37^{-/-}$ as well as in $Cx40^{-/-}$ platelets, i.e., fibrinogen binding and α-granule secretion were decreased, even under conditions when direct platelet-platelet contacts were excluded (Vaiyapuri et al., 2012, 2013). This implies that the contribution of connexins to platelet aggregation may not be limited to the phase during which platelets come into stable contact with each other but might also occur when only HCs are present. The mechanism by which Cx37/Cx40 HCs open in the presence of relative high extracellular Ca^{2+} in blood (which normally keep HCs closed) still remains to be determined. Connexin HCs may allow for the release of ATP from activated platelets, which then might act in an autocrine/paracrine manner on P2X1, inducing a further increase in platelet activation state. The recent discovery of Panx1 in human platelets is in this respect of particular interest (Taylor et al., 2014; Molica et al., 2015).

C. Endothelial Connexins in Ischemia-Reperfusion Injury

It is increasingly recognized that deleterious consequences of ischemia-reperfusion are influenced by the dysfunction of the endothelium and the extent of neutrophil infiltration. Although the crucial role of endothelial connexins in diseases of large arteries is now well established, surprising little attention has been given to the role of endothelial connexins, i.e., Cx40 and Cx37, in the response of the microcirculation to ischemia-reperfusion injury (Fig. 6). Interestingly, spontaneous recovery of tissue perfusion after severe unilateral hindlimb ischemia was reduced, and the survival of distal limb tissue was compromised in $Cx40^{-/-}$ mice (Fang et al., 2012). The poor recovery from the ischemic insult in $Cx40^{-/-}$ mice appeared to be due to compromised regulation of tissue perfusion, vascular remodeling, and a prolonged inflammatory response (Fang et al., 2013). Thirty minutes of ischemia followed by 24-hour reperfusion resulted in increased myocardial infarct size in mice with endothelial-specific deletion of Cx40; however, no deleterious effects were found in mice with Cx37 deletion (Morel et al., 2014a).

Mechanistically, endothelial Cx40-dependent cardioprotection appeared to involve CD73 activation that, in turn, limited neutrophil infiltration after cardiac reperfusion (Morel et al., 2014a). The Cx40-CD73 axis may thus represent a novel pharmacological target for controlling the damage associated with reperfusion in coronary disease.

The antiarrhythmic peptide rotigaptide or its analog danegaptide (see *section III.B.3*) reduce myocardial ischemia-reperfusion injury in procedures on large animals such as open-chest dogs (Hennan et al., 2006) and when given at the moment of reperfusion in pigs (Skyschally et al., 2013; Pedersen et al., 2016). Although the cardioprotective effects of these peptides are typically ascribed to the maintenance of Cx43-mediated GJ intercellular communication between cardiomyocytes, a recent translational study suggested additional effects of these peptides on the human endothelium. By measuring forearm arterial blood flow in humans, endothelium-dependent vasodilation in response to acetylcholine was attenuated after ischemia-reperfusion in the presence of placebo but not in the presence of intra-arterial rotigaptide (Pedersen et al., 2016).

D. Connexins in Restenosis

Acute vascular injury induced by balloon angioplasty is associated with increased Cx43 expression in neointimal smooth muscle cells and macrophages in various animal models (Polacek et al., 1997; Yeh et al., 1997; Plenz et al., 2004; Wang et al., 2005; Chadjichristos et al., 2006; Li et al., 2012b). Although treatment with the cholesterol-lowering lovastatin or the angiotensin-converting enzyme inhibitor ramipril was shown to decrease both Cx43 expression and neointima formation after balloon injury, a causal link remained to be proven (Wang et al., 2005; Li et al., 2012b). Thus, hypercholesterolemic $Cx43^{+/-}Ldlr^{-/-}$ mice and $Cx43^{+/+}Ldlr^{-/-}$ controls were subjected to carotid balloon distension injury in vivo, which induced marked endothelial denudation and activation of medial smooth muscle cells (Chadjichristos et al., 2006). This genetic reduction in Cx43 expression was found to limit neointima formation after the acute vascular injury by decreasing the inflammatory response as well as reducing smooth muscle cell migration and proliferation. Moreover, endothelial repair was enhanced in mice with reduced Cx43 (Chadjichristos et al., 2006). This suggests that Cx43 might be an attractive target for local delivery strategies aimed at reducing restenosis. Interestingly, effective knock-down of Cx43 was recently achieved with a lentiviral vector expressing Cx43-targeting shRNA and also resulted in an attenuation of neointima formation after balloon injury in rats (Han et al., 2015). Surprisingly, smooth muscle-targeted knockout of Cx43 enhanced neointima formation in response to carotid wire or occlusion injury (Liao et al., 2007). Thus, the level to which Cx43 is reduced as well as the progression of the disease may be of crucial importance for the final outcome. Additional research will be needed before moving on to a translational setting of targeting Cx43 for the reduction of restenosis.

V. Connexins in Cardiac Disease

The heart mainly expresses the connexin isoforms Cx31.9, Cx37, Cx40, Cx43, and Cx45, whereby Cx43 is the most abundant isoform and is mainly found in ventricular cardiomyocytes (Severs et al., 2008) but is also expressed in fibroblasts (McArthur et al., 2015) and stem cells (Lu et al., 2012b). Other isoforms like Cx40 are expressed in the atria of the heart; Cx31 and Cx45 in the conductance system; and Cx37, Cx40, and Cx43 in the coronary circulation. The expression of Cx43 in cardiomyocytes is affected by many factors, including factors released by activation of the neurohumoral system (Salameh et al., 2013) or immune system (Zhang et al., 2016a) as well as by microRNAs (miRNA; further discussed in *section V.B.1*). As introduced earlier, connexins have a fast protein turnover with a half-life in the order of 1–2 hours in cardiomyocytes, necessitating specific requirements in the organization of the connexin turnover cycle (see *section II.B*). As a result, the high level of forward connexin trafficking to the sarcolemma needs to be balanced by a well-organized degradation machinery that encompasses multiple proteolytic (Beardslee et al., 1998) and lysosomal (Laing et al., 1997) pathways. Forward trafficking of connexins to the sarcolemma is a crucial component of the connexin life cycle in cardiomyocytes, and in the human heart it has been demonstrated that four truncated connexin isoforms with a molecular weight of ~20 kDa play a central role because their ablation arrests trafficking of full-length connexin (Smyth and Shaw, 2013). At the intercalated discs, Cx43 interacts with various scaffolding proteins including ZO-1 and others (see *section II.B.3*). Transmembrane protein 65 (Tmem65) is another scaffolding protein, which functionally regulates Cx43 and thereby cellular coupling (Sharma et al., 2015). The end-station of forward trafficking is the perinexus, a zone at the periphery of the nexus that contains the GJ plaques at the intercalated disks (IDs) (Rhett et al., 2011; Rhett and Gourdie, 2012). In the perinexus, Cx43 arrives as HCs, which then come loose of their ZO-1 binding to become incorporated into the GJs. The perinexus as a defined zone of HC residency is likely to be the site where HC opening may occur. HC opening can be triggered by lowering $[Ca^{2+}]_e$, increasing $[Ca^{2+}]_i$, connexin dephosphorylation, metabolic inhibition, or hyperosmolar conditions, as demonstrated in isolated ventricular cardiomyocytes by dye uptake and electrophysiological methods (Kondo et al., 2000; John et al.,

2003; Wang et al., 2012a). Within cardiomyocytes, Cx43 is located also in subsarcolemmal mitochondria (see below) and the CT of Cx43 translocates into the nucleus and inhibits cell proliferation (Dang et al., 2003; Zhao et al., 2015). Cardiac connexins also have extensive non-channel functions that in many cases link to interactions of the Cx43 CT with various scaffolding and signaling proteins, forming a connexin interactome network or "connexome." Cx43 interacts with proteins related to various biologic processes such as cell cycle, metabolism, signaling, and trafficking (see also *section II.C*). Importantly, the interactome of Cx43 is differentially modulated in diseased hearts (for review, see Martins-Marques et al., 2015a). Work of the Delmar group has demonstrated that the Cx43 CT is involved in interactions that influence capture of the microtubule plus end at the ID and thereby facilitate sarcolemmal delivery of Na^+ channels at the cell end (Agullo-Pascual et al., 2014b; reviewed in Leo-Macias et al., 2016). Thus, alterations in connexin trafficking are likely to influence cell-cell coupling as well as electrical excitability, thereby leading to arrhythmia. Along this line, the Cx43 interactome partner plakophilin-2, which associates with Cx43 at the ID has been demonstrated to be involved in arrhythmias associated with the Brugada syndrome in genetically predisposed patients (Cerrone et al., 2014; Agullo-Pascual et al., 2014a). Figure 7 summarizes some of the proposed roles of GJs and HCs in cardiac ischemia-reperfusion injury.

A. Cx43 in Mitochondria

Cx43 is detected in cardiomyocyte mitochondria from mouse, rat, porcine, and human origin using antibody-dependent and -independent techniques (Boengler et al., 2005; Miro-Casas et al., 2009; Jovic et al., 2012). The analysis of subsarcolemmal and interfibrillar mitochondria for the presence of Cx43 shows that Cx43 is almost exclusively localized in subsarcolemmal mitochondria (Boengler et al., 2009; Sun et al., 2015b). Cx43 is encoded in the nuclear genome and imported into the mitochondria in a heat shock protein 90, translocase of the outer membrane 20-dependent fashion. Cx43 is located at the inner membrane of subsarcolemmal mitochondria, with its carboxy terminus oriented toward the intermembrane space (Rodríguez-Sinovas et al., 2006), but may also be present at the outer mitochondrial membrane regulating the release of some intermembrane space proteins (Goubaeva et al., 2007).

In the inner membrane of subsarcolemmal mitochondria, Cx43 interacts with the apoptosis-inducing factor, the β-subunit of the electron-transfer protein (Denuc et al., 2016) and the ATP-sensitive potassium channel subunit Kir6.1 (Waza et al., 2014). The amount of mitochondrial Cx43 declines in cardiomyocytes after the activation of the *N*-methyl-D-aspartate receptor 1 by the enhanced mitochondrial translocation of the matrix-metalloproteinase 9 (Tyagi et al., 2010). In isolated mitochondria of Cx43-deficient mice hearts, the reduced mitochondrial Cx43 content is associated with a switch of the mitochondrial NOS isoform and the decrease in mitochondrial nitric oxide formation (Kirca et al., 2015).

In isolated subsarcolemmal mitochondria, chemical cross-linking generates Cx43-dependent protein-complexes at a molecular weight corresponding to that of GJ-enriched membranes, possibly representing Cx43

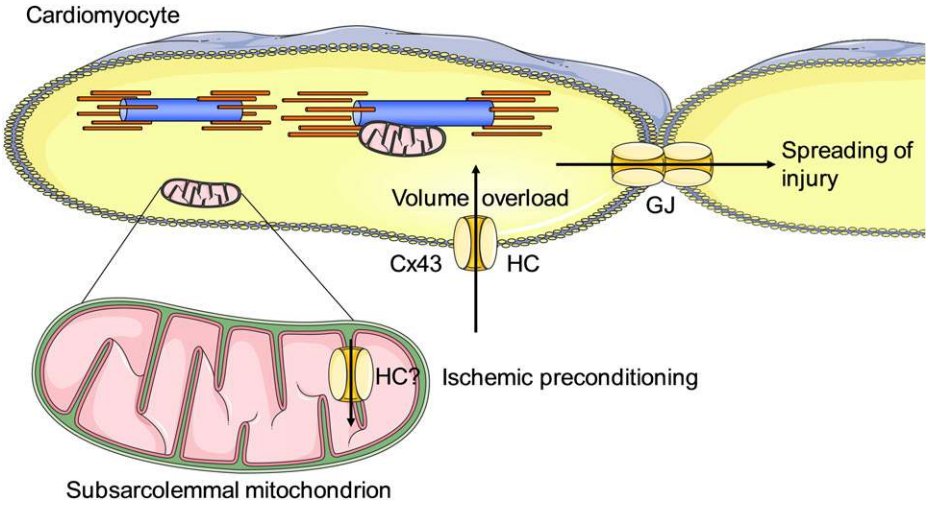

Fig. 7. Roles of Cx43 in cardiac ischemia-reperfusion. Gap junctions close under ischemic conditions ("healing over" caused by the low pH and elevated $[Ca^{2+}]_i$) but substantial coupling may persist after ischemia-reperfusion (Ruiz-Meana et al., 2001). Open GJs may act beneficially by supplying essential metabolites to neighboring cells but may also spread injury signals, causing cell death propagation (reviewed in Decrock et al., 2009b; Michela et al., 2015). GJs have been implicated in the spreading of hypercontracture necrosis in a process mediated by Na^+ flux through GJs (reviewed in García-Dorado et al., 2004). Uncontrolled hemichannel (HC) opening facilitates ionic fluxes that may lead to cell swelling (Wang et al., 2013c). Cx43 is also present in mitochondria where they are involved in the signaling cascade of ischemic preconditioning, conferring cardioprotective effects (reviewed in Schulz et al., 2007; Miura et al., 2010; Schulz et al., 2015). Mitochondrial Cx43 has been demonstrated to form hexameric structures involved in inner mitochondrial membrane K^+ fluxes, pointing to functional HCs (Miro-Casas et al., 2009).

hexamers. Mitochondrial uptake of the Cx43-formed channel permeable dye Lucifer yellow, which is inhibited by the chemically unrelated Cx43-formed channel blockers carbenoxolone and heptanol, suggests a functional Cx43-formed channel within the inner membrane of subsarcolemmal mitochondria (Miro-Casas et al., 2009; Soetkamp et al., 2014). The finding that the administration of the Cx43 HC-specific peptide Gap19 as well as the genetic ablation of Cx43 reduce the potassium influx into the mitochondrial matrix further strengthens the hypothesis that Cx43-formed channels are present in the inner membrane of subsarcolemmal mitochondria (Miro-Casas et al., 2009; Soetkamp et al., 2014). Subsarcolemmal mitochondria are more responsive than interfibrillar mitochondria to fibroblast growth factor-2–triggered protection from calcium-induced permeability transition pore opening by a mitochondrial Cx43 channel-mediated pathway (Srisakuldee et al., 2014).

Apart from ion fluxes, both a pharmacological inhibition and a genetic ablation of Cx43 reduce mitochondrial complex 1-mediated oxygen consumption and ATP production, whereas complex 2-mediated respiration is not affected. Possibly, Cx43 interacts with proteins of complex 1 of the electron transport chain (Boengler et al., 2012; Denuc et al., 2016). Mitochondria are central to ROS formation and a relationship between Cx43 expression and ROS formation has been established (Matsuyama and Kawahara, 2011; Denuc et al., 2016), whereby Cx43-formed channel inhibition or Cx43 downregulation decrease the amounts of ROS formation.

It is not yet known whether all Cx43 phosphorylation sites described in *section II.F* are also phosphorylated in mitochondrial Cx43. However, phosphorylation of mitochondrial Cx43 is found at Ser-262 in rat subsarcolemmal mitochondria (Srisakuldee et al., 2014), whereas Cx43 phosphorylation at Ser-368 is detected in mouse, rat, and porcine subsarcolemmal mitochondria (Boengler et al., 2011; Srisakuldee et al., 2014; Shan et al., 2015). Phosphorylation of Cx43 is not necessarily achieved outside the mitochondria, since PKC is present in mitochondria and the stimulation of such mitochondrial PKC leads to Cx43 phosphorylation at Ser-262 and Ser-368 in rat subsarcolemmal mitochondria (Srisakuldee et al., 2014). Activation of mitochondrial PKC renders mitochondria more tolerant toward Ca^{2+} overload (Srisakuldee et al., 2014).

In addition to the well documented protective role of Cx43 in cardiac subsarcolemmal mitochondria, recent evidence indicates that mitochondrial Cx43 may also contribute to cardiac injury and cell death. Gadicherla et al. (2017) demonstrated that Cx43 forms functional hemichannels in cardiac subsarcolemmal mitochondria, which contribute to Ca^{2+} entry and trigger permeability transition and cell injury/cell death (Gadicherla et al., 2017). Compared to Gap26 and Gap19, RRNYRRNY peptide appeared to most active in inhibiting mitochondrial Cx43 hemichannel activity. The RRNYRRNY peptide also strongly reduced the infarct size in ex vivo cardiac ischemia-reperfusion studies (Gadicherla et al., 2017). Thus, Cx43 in cardiac subsarcolemmal mitochondria may protect in the context of ischemic preconditioning, but in the absence of preconditioning, it contributes to cardiac injury provoked by ischemia.

B. Cx43 and Risk Factors of Cardiovascular Diseases

1. Age and Sex. In the promotor region of the Cx43 gene, a series of half-palindromic estrogen response elements is present. In HeLa cells transfected with a luciferase-connexin43 promoter fusion construct, an upregulation of luciferase expression by estrogen occurs (Yu et al., 1994). Although Cx43 mRNA levels in ventricular tissue from neonatal male and female littermates are similar, the Cx43 mRNA levels are higher in adult female compared with age-matched male hearts (Rosenkranz-Weiss et al., 1994). Interestingly, phenylephrine treatment increases Cx43 expression only in female cardiomyocytes (Stauffer et al., 2011). Also on the protein level, expression of Cx43 is markedly lower in males of both normotensive and hypertensive rats compared with female rats (Knezl et al., 2008). The difference in Cx43 expression between males and females is predominantly seen at the level of GJs (Thomas et al., 2011). Multiple reports have documented that Cx43 protein expression declines with age in the hearts of hamsters (Chen and Jones, 2000), guinea pigs (Jones et al., 2004; Jones and Lancaster, 2015), mouse (Bonda et al., 2016), rats (Watanabe et al., 2004; Lancaster et al., 2011), and rabbits (Yan et al., 2013). Such reduced expression with age is associated with enhanced lateralization of the protein as observed in rat and rabbit hearts (Dhein and Hammerath, 2001; Fannin et al., 2014). Interestingly, Cx43 expression remains higher in aged (16 months) female compared with aged male rat hearts (Tribulova et al., 2005). Cx43 expression is reduced in the myocardium of postmenopausal or ovariectomized rats and such decline is attenuated by estrogen supplementation (Wang et al., 2015b) or activation of protein kinase C (Lancaster et al., 2011). While in animal experiments Cx43 expression appears to be higher in female compared with male hearts, the Cx43 expression is higher in male than in female and higher in epicardial than in endocardial tissue samples from nondiseased human transplant donor hearts (Gaborit et al., 2010). Enhanced lateralization of Cx43, however, is detected in human tissue of aged patients (with atrial fibrillation), and this correlates with an increased incidence of atrial fibrillation (Kostin et al., 2002); similarly the age-dependent occurrence of arrhythmias in rat hearts is associated with lateralization of Cx43 (Fannin et al., 2014).

Recently, microRNAs (miR) have been found to regulate Cx43 expression within cardiomyocytes,

including miR-1 (Xu et al., 2012) [which is higher expressed in males than in females, possibly explaining the sex difference in Cx43 expression (Stauffer et al., 2011)], miR-19a/b (Danielson et al., 2013), miR-23a (Wang et al., 2015b) (regulated via estrogen), and miR-130a (Osbourne et al., 2014) (also found in fibroblasts).

Finally, in aged mouse hearts, not only the amount of gap junctional Cx43 but also that of mitochondrial Cx43 is reduced (Boengler et al., 2007).

2. Hypertension and Hypertrophy. In spontaneously hypertensive rats, Cx43 protein expression either declines (Bačová et al., 2012; Benova et al., 2013; Zhang et al., 2014) or increases (Chen et al., 2007; Zhao et al., 2008; Radosinska et al., 2013). The differences between the aforementioned studies may be due to the degree of left ventricular hypertrophy associated with hypertension, because in porcine and human myocardium Cx43 expression increases in the compensated phase but gradually decreases with the progression/decompensation of hypertrophy (Formigli et al., 2003; Kostin et al., 2004).

In mice, pressure overload by transverse aortic constriction reduces the expression of Cx43 due to increased ubiquitination; this effect is blunted by knockdown of AMP kinase (Alesutan et al., 2015). When the ubiquitinase Wwp1 is globally overexpressed in mice, the highest induction of Wwp1 expression is observed in the heart associated with a 90% reduction in cardiac Cx43 protein expression, left ventricular hypertrophy, and the development of lethal ventricular arrhythmias (Basheer et al., 2015). Cardiomyocyte-specific overexpression of calcineurin induces cardiomyocyte hypertrophy and downregulation of Cx43 expression and phosphorylation (Sun et al., 2015c). Myocardial Cx43 expression is also significantly decreased and redistributed after hypertrophy induced by prolonged isoproterenol treatment in rats in vivo. The decrease and redistribution of Cx43 is reduced after treatment with the ATP-dependent K^+ channel agonist nicorandil (Sun et al., 2015c). In contrast, rats with left ventricular hypertrophy secondary to transverse aortic constriction showed a decrease in miR-1 and an increase of Cx43 expression and phosphorylation at epitopes displacing Cx43 from the GJs (Curcio et al., 2013).

Interventions known to reduce hypertension and/or hypertrophy such as blockade or deletion of angiotensin II receptor I (Zhao et al., 2008; Yasuno et al., 2013), aldosterone antagonists (Qu et al., 2009), or renin inhibition (Zhang et al., 2014) prevent the changes in Cx43 expression, phosphorylation, or lateralization seen with hypertension and/or hypertrophy. Interestingly, melatonin also attenuates abnormal myocardial Cx43 distribution, upregulates Cx43 mRNA and protein expression, and maintains Cx43 phosphorylation forms in spontaneously hypertensive rats (Benova et al., 2013). With hypertension secondary to abdominal aortic constriction, Cx43 expression is reduced and Cx43 is displaced from the intercalated discs (Emdad et al., 2001), the effect being reversed by application of a H2S donors (Huang et al., 2012b).

The expression of mitochondrial Cx43 in hypertension and hypertrophy has not been studied up to now.

3. Diabetes and Hypercholesterolemia. In a prediabetes model in rats, Cx43 expression is unaltered and the fraction of Cx43 localized at the intercalated discs is increased (Axelsen et al., 2015). Cx43 mRNA and protein levels are either reduced (Lin et al., 2006a,b; Veeranki et al., 2016), unchanged (Nygren et al., 2007; Stables et al., 2014), or elevated (Howarth et al., 2008; Radosinska et al., 2013; Anna et al., 2014; Joshi et al., 2015) with diabetes. Enhanced lateralization of Cx43 is observed with diabetes in rat hearts with unaltered (Nygren et al., 2007) or increased expression of Cx43 (Radosinska et al., 2013; Anna et al., 2014; Joshi et al., 2015). The decreased amount of Cx43 in obese-diabetic mice is restored by moderate exercise (Veeranki et al., 2016) and the lateralization of Cx43 is normalized by omega-3-polyunsaturated fatty acid administration (Anna et al., 2014). With diabetes, overall (Radosinska et al., 2013) and tyrosine (Joshi et al., 2015) Cx43 phosphorylation is decreased, but increased Cx43 phosphorylation has also been described (Lin et al., 2006b; Anna et al., 2014; Palatinus and Gourdie, 2016).

In rabbits fed a high-cholesterol diet, the expression of Cx43 is increased in left atria (Lee et al., 2012), but is decreased in left ventricular tissue, where also the lateralization of Cx43 is enhanced (Lin et al., 2005). Such disturbed distribution of Cx43 is also found in rats fed a high-fat diet; however, in contrast to rabbits, the left ventricular expression of Cx43 remains unchanged, whereas the mitochondrial amount of Cx43 is decreased (Görbe et al., 2011).

4. Postmyocardial Infarction. Overall Cx43 expression decreases and gap junctional localization of Cx43 is disturbed, i.e., lateralized, postmyocardial infarction in the border and infarct zones of mouse (Lindsey et al., 2006; Jackson et al., 2008), rat (Matsushita et al., 1999; Savi et al., 2016), and dog (Peters et al., 1997) hearts. Wall stretch can influence Cx43 expression and localization either directly or indirectly via angiotensin II (Zhuang et al., 2000; Saffitz and Kleber, 2004; Hussain et al., 2010; Salameh et al., 2010). The Cx43 expression after myocardial infarction is preserved in matrix-metalloproteinase 7-deficient mice, indicating that Cx43 represents a matrix-metalloproteinase 7 substrate (Lindsey et al., 2006). The lateralization of Cx43 is limited when mice receive omega-3 fatty acids before myocardial infarction (Baum et al., 2012). The transplantation of cardiac stem cells elevates Cx43 expression after myocardial infarction in rat hearts (Hou et al., 2015), and also the combined administration of hepatocyte growth factor and insulin-like growth factor improves Cx43 expression and intercellular coupling (Savi et al., 2016). The role of mitochondrial Cx43 expression

postmyocardial infarction has not been studied so far. Further details on postmyocardial infarction Cx43 alterations in the context of their arrhythmogenic consequences is given in *section VI*.

5. Heart Failure. The Cx43 expression is reduced in a pacing-induced heart failure model in rabbits (Ai and Pogwizd, 2005). Here, the overexpression of Cx43 in cardiomyocytes from failing hearts to protein levels comparable to that of control cells improves cell coupling (Ai et al., 2010). Additionally, total Cx43 protein expression decreases in failing mice (Danielson et al., 2013), rat (Wang et al., 2012b; dos Santos et al., 2016), and dog (Akar et al., 2004) hearts. Moreover, a heterogeneous distribution of Cx43 at the plasma membrane is observed in patients with ischemic and/or dilated end-stage heart failure (Dupont et al., 2001; Kostin et al., 2003) resulting in a proarrhythmic potential (Glukhov et al., 2012) (for further discussion see *section VI*). The analysis of the phosphorylation status of Cx43 shows either dephosphorylation (Akar et al., 2004; Ai and Pogwizd, 2005) or phosphorylation at Ser-255 (Sato et al., 2008). Dephosphorylation of Cx43 in pacing-induced heart failure in rabbits is associated with increased activation of p21-activated kinase 1 and subsequently protein phosphatase 2A. In isolated cardiomyocytes, Cx43 dephosphorylation is abolished by inhibition of protein phosphatase 2A (Ai et al., 2011). The induction of mitophagy and matrix metalloproteinase activity is suggested to contribute to the decreased expression of Cx43 in heart failure (Givvimani et al., 2014), and the inhibition of the proteasome increases Cx43 amounts in adriamycin-induced heart failure in rats (Chen et al., 2015a).

Data on the role of mitochondrial Cx43 in heart failure are limited. One study addressed the function of mitochondrial Cx43 in a model of doxorubicin cardiotoxicity in rat hearts and H9C2 cells (Pecoraro et al., 2015). Doxorubicin induces mitochondrial translocation of Cx43 in a heat shock protein 90-dependent manner, and blocking the Cx43 mitochondrial import increases ROS formation and mitochondrial calcium content and enhances depolarization of the mitochondrial membrane compared with doxorubin treatment alone. Thus, mitochondrial Cx43 appears to be important for the protection against doxorubin-induced cardiotoxicity. In a second study, the induction of dilated cardiomyopathy by furazolidone decreases the amount of Cx43 in both the myocardium and isolated mitochondria (Shan et al., 2015) and increased mitochondrial Cx43 is dephosphorylated at Ser-368. The functional importance of these mitochondrial changes remains unknown.

C. Cx43 in Ischemia-Reperfusion Injury

Cx43-formed HCs open upon metabolic inhibition (Kondo et al., 2000; Shintani-Ishida et al., 2007) or ischemia (Johansen et al., 2011), leading to release of ATP (Clarke et al., 2009), changes in $[Ca^{2+}]_i$ and $[Na^+]_i$ (Li et al., 2001), and finally cell swelling and rupture of the sarcolemma (for review, see Garcia-Dorado et al., 2012). Accordingly, HC inhibition by 18-αGA or the connexin mimetic peptide Gap26 is protective in neonatal cardiomyocytes after simulated ischemia (Shintani-Ishida et al., 2007; Clarke et al., 2009). The use of the specific HC inhibitor Gap19 protects cardiomyocytes from volume overload, increases cell viability, and reduces infarct size after ischemia- reperfusion (Wang et al., 2013c).

The use of less HC-specific inhibitors such as Gap26/Gap27 also reduces myocardial infarction after ischemia-reperfusion (Hawat et al., 2010, 2012), and mice in which Cx43 was replaced by Cx32 have smaller infarcts than wild-type mice (Rodríguez-Sinovas et al., 2010). Although in $Cx43^{+/-}$ mice myocardial infarction after ischemia-reperfusion is either unchanged (Schwanke et al., 2002; Heinzel et al., 2005; Sanchez et al., 2013) or reduced (Kanno et al., 2003), any further reduction of the Cx43 expression in conditional knockout mice results in smaller infarcts after ischemia-reperfusion (Sanchez et al., 2013).

Cx43 phosphorylation and dephosphorylation during ischemia-reperfusion is closely associated with cellular ATP levels (Turner et al., 2004). In particular, ischemia or hypoxia induce dephosphorylation of Cx43 at Ser-365 (Solan et al., 2007) or at Ser-325/328/330 (Lampe et al., 2006), but also Cx43 phosphorylation at Ser-262, Ser-368, or Ser-373 (Ek-Vitorin et al., 2006; Dunn and Lampe, 2014; Smyth et al., 2014). Ser-365 is suggested to function as a "gatekeeper," since Ser-365 phosphorylation prevents Ser-368 phosphorylation (Solan et al., 2007) (see *section II.F.4.b*). The interaction between Cx43 and ZO-1 is regulated by phosphorylation at the Akt target site Ser-373, whereby the phosphorylation of Cx43 at Ser-373 disturbs the interaction between the two proteins, resulting in increased size of GJs and enhanced cell-cell communication (Dunn and Lampe, 2014). The mutation of Ser-373 to phosphorylation-insensitive Ala reduces the interaction between Cx43 and 14-3-3- proteins, stabilizing Cx43 at the plasma membrane and thereby avoiding Cx43 degradation (Smyth et al., 2014). During ischemia, a redistribution of Cx43 occurs from the intercalated disks to the lateral sides of the cardiomyocytes (Beardslee et al., 2000). Such lateralized Cx43 is mainly dephosphorylated, whereas phosphorylated Cx43 remains at the GJs (Severs et al., 2008). Recent data show that ischemia induces Cx43 ubiquitination at the intercalated disks and subsequent autophagic degradation of the protein requiring both AMP kinase (during ischemia) and Beclin 1 (during reperfusion) (Martins-Marques et al., 2015b,c). Ubiquitination of Cx43 is decreased by the mutation of the phosphorylation site Ser-373 to Ala (Smyth et al., 2014). Also matrix-metalloproteinase 9 appears to be involved in the hypoxia-induced degradation of Cx43 in H9c2 cells (Wu et al., 2013).

In H9C2 cells subjected to 12-hour hypoxia, the phosphorylation of mitochondrial Cx43 is increased; however, no specific phosphorylation sites have been tested (Waza et al., 2014). After a shorter phase of ischemia (30-minute ischemia, 5-minute reperfusion), an overall dephosphorylation of Cx43 is observed in rat myocardial mitochondria (Görbe et al., 2011). Also, in porcine myocardial mitochondria, ischemia induces a dephosphorylation at the PKC-target site of Cx43, namely Ser-368 (Totzeck et al., 2008).

Several pharmacological interventions are known to reduce infarct size after ischemia-reperfusion and some of them impact on the phosphorylation status of Cx43. Metabolic inhibition significantly reduces dye coupling of cardiomyocytes that is prevented by pretreatment with estradiol; the beneficial effect of estradiol is abolished by tamoxifen, a potent estrogen receptor antagonist. Double immunofluorescence microscopy shows that metabolic inhibition induces the accumulation of nonphosphorylated Cx43 at GJs and that this is prevented by estradiol pretreatment. Inhibition of protein kinase C with chelerythrine blocks the estrogen-induced increase of phosphorylated Cx43 (Chung et al., 2004).

In canine hearts, administration of 17β-estradiol decreases myocardial infarction, and this effect is associated with a decrease in the overall phosphorylation of Cx43 (Lee et al., 2004). Also, inhibition of p38 MAPK induces phosphorylation of Cx43 at Ser-368 (Surinkaew et al., 2013). The sphingosine-1 phosphate (S1P) constituent of high-density lipoproteins limits cell death by ischemia-reperfusion, and this is accompanied with an increased Cx43 phosphorylation at Ser-368. A causal role for this phosphorylation site in cardioprotection by sphingosine-1 phosphate is shown in mice, in which Ser-368 of Cx43 is mutated to Ala; here, the infarct size reduction by sphingosine-1 phosphate is lost (Morel et al., 2016). Enhanced phosphorylation of Cx43 (total and mitochondrial) at Ser-262 and Ser-368 is achieved in isolated rat hearts by diazoxide or fibroblast growth factor 2 (FGF-2) (Srisakuldee et al., 2009, 2014). In neonatal cardiomyocytes, the overexpression of Cx43, in which Ser-262 is mutated to alanine, results in augmented cell death after simulated ischemia (Srisakuldee et al., 2009).

D. Cx43 and Cardioprotection

The phenomena of endogenous conditioning describe the infarct size reduction after sustained ischemia-reperfusion by brief nonlethal periods of ischemia-reperfusion, which are performed either before (pre-), during (per-), or after (post-) the sustained phase of ischemia followed by reperfusion. The protection by such endogenous conditioning is often attenuated or lost in the presence of cardiovascular risk factors or diseases (Ferdinandy et al., 2014), where total and mitochondrial Cx43 expression, localization, and/or phosphorylation is altered. Ischemic preconditioning (IPC) suppresses chemical coupling of cardiomyocytes via GJs during ischemia. By using a protocol of simulated ischemia-reperfusion in vitro, only wild-type cardiomyocytes are protected by IPC, whereas cardiomyocytes isolated from $Cx43^{+/-}$ animals show no preservation of cell viability (Li et al., 2004a). In $Cx43^{+/-}$ animals, the infarct size reduction by IPC is abolished (Schwanke et al., 2002), whereas noninduced conditional Cx43 knockout mice (also having a 50% reduction of Cx43) demonstrate cardioprotection by IPC (Sanchez et al., 2013). The further reduction of the Cx43 content in these mice; however, abolishes IPC-induced cardioprotection (Sanchez et al., 2013). In addition, in mice, in which Cx43 is replaced by Cx32, IPC is no longer effective (Rodríguez-Sinovas et al., 2010). When Cx43-formed channels are inhibited by heptanol during the preconditioning cycles of ischemia and reperfusion infarct size reduction by IPC is lost (Li et al., 2002).

The analysis of the phosphorylation status of Cx43 reveals that IPC prevents the ischemia-induced dephosphorylation of Cx43 in rat hearts in vitro (Jain et al., 2003) and in vivo (Hatanaka et al., 2004) or in pig hearts in vivo (Ahlquist et al., 1989; Totzeck et al., 2008); in one study, however, IPC had no impact on Cx43 phosphorylation (Mühlfeld et al., 2010). Preserved Cx43 phosphorylation is in part due to the increased activity of PKC, since knockout of PKC not only abrogates the protection but also the maintained Cx43 phosphorylation by IPC (Hund et al., 2007).

IPC not only affects gap junctional, but also mitochondrial Cx43. The amount of the protein within mitochondria is increased upon IPC in isolated mouse (Sun et al., 2015b) and rat hearts (Boengler et al., 2005; Soetkamp et al., 2014) and pig hearts in vivo (Boengler et al., 2005). In bone marrow stem cells, the overexpression of specifically mitochondrial Cx43 is sufficient to confer cytoprotection (Lu et al., 2010), pointing to the important role of mitochondrial Cx43 in cell survival.

Pharmacological preconditioning with diazoxide augments mitochondrial Cx43 in a model of hypothermia (Yang et al., 2011). When the mitochondrial import of Cx43 is blocked by the use of the Hsp90-inhibitor geldanamycin, the infarct size reduction by diazoxide is lost. Since in this model the gap junctional content of Cx43 remains unaffected, the loss of cardioprotection by diazoxide is associated with the reduced content of mitochondrial Cx43. In addition to Cx43, geldanamycin also inhibits the mitochondrial import of PKC_{ε}, which may phosphorylate mitochondrial Cx43 (Budas et al., 2010). The analysis of the mechanisms by which mitochondrial Cx43 contributes to diazoxide-induced cardioprotection demonstrates that the generation of reactive oxygen species, which, if present in low amounts, function as trigger molecules of preconditioning, is reduced in cardiomyocytes isolated from

Cx43-deficient mice (Heinzel et al., 2005). Furthermore, IPC induces *S*-nitrosation, an important posttranslational modification in cardioprotection (Lin et al., 2009), predominantly of subsarcolemmal mitochondrial proteins (Sun et al., 2015b). One protein with enhanced amounts of *S*-nitrosation after IPC is mitochondrial Cx43, and the *S*-nitrosation of mitochondrial Cx43 enhances the permeability of subsarcolemmal mitochondria toward potassium ions and increases reactive oxygen species formation (Soetkamp et al., 2014). Subsarcolemmal mitochondria isolated from wild-type mice show increased respiration when the isolated mitochondria undergo IPC; however, in mice in which Cx43 is replaced by Cx32, such preserved oxygen consumption is lost (Ruiz-Meana et al., 2014). In addition, mitochondrial Cx43 may contribute to cardioprotection via its effect on permeability transition pore opening (see above), because the inhibition of transition pore opening reduces myocardial infarction (Ong et al., 2015).

In remote ischemic preconditioning, where the short periods of ischemia-reperfusion occur in organs other than the heart, sustained Cx43 expression and phosphorylation is observed in hearts after ischemia-reperfusion (Brandenburger et al., 2014).

Ischemic postconditioning induces Cx43 phosphorylation in rat hearts in vivo (Wu et al., 2012; He et al., 2015), but also in mitochondria isolated from mice hearts that were subjected to a postconditioning protocol (Boengler et al., 2011). However, one study demonstrated reduced phosphorylation of mitochondrial Cx43 after postconditioning compared with ischemia-reperfusion alone (Penna et al., 2009). Nevertheless, altered Cx43 phosphorylation is not important for the cardioprotection by postconditioning, since $Cx43^{+/-}$ mice still have smaller infarcts than wild-type mice upon a postconditioning stimulus (Heusch et al., 2006).

Pharmacological cardioprotection by induction of HO-1 protects the rat heart against postischemic dysfunction and arrhythmias by a Cx43-dependent mechanism (Lakkisto et al., 2009).

Taken together, therapeutic activation of Cx43 expression/function may provide a novel tool for cardioprotection in ischemic heart disease.

VI. Connexins and Arrhythmias

The heart is a rhythmically beating organ in which synchronized contraction is achieved by the organized propagation of action potentials between cells. The normal heart rhythm requires a pacemaker region, the sinus node, from which the electrical activity is transferred to the atrium, then the atrioventricular node, the bundle of His, the Tawara branches, and (via the Purkinje fibers) the working ventricular myocardium. Within the ventricular conduction system, the action potential propagates with a velocity of about 0.8–1.0 m/s, whereas the conduction velocity within the working myocardium is about 0.4 m/s along the fibers and 0.2 m/s transverse to the fiber axis. The regional and directional differences in conduction velocity, termed anisotropy, are basic characteristics of cardiac tissue and can be either uniform or nonuniform (Spach and Dolber, 1990). Successful and sustained coupling requires >38 GJ channels per cell, whereas conduction block occurs if less than 13 are present. In addition to supporting the role of GJs in cardiac cell coupling, the classic experiments of Weingart and Maurer (1988) excluded the possibility that action potentials can be transferred via ephaptic coupling (electrical field effects) or local ionic interactions at the level of closely appositioned membranes. However, under certain pathophysiological conditions, such phenomena may contribute to conduction between cardiomyocytes (Veeraraghavan et al., 2015; George et al., 2016; George and Poelzing, 2016).

The different anisotropic and conductive characteristics of different cardiac tissues derive, in part, from differences in the connexin isoforms expressed and the distributions of GJs (Saffitz et al., 1992, 1994; Davis et al., 1994; Saffitz et al., 1994).

Targeted ablation of several connexins in mice has demonstrated their importance for electrical coupling in various cardiovascular tissues and has shown that their absence can lead to various arrhythmias or sudden death (Kirchhoff et al., 1998; Simon et al., 1998; Gutstein et al., 2001a,b; Kreuzberg et al., 2006). Such studies also revealed that a reduction in Cx43 expression of about 50% is required before conduction velocity is reduced (Guerrero et al., 1997; Thomas et al., 1998; Morley et al., 1999; Morley et al., 2000). However, the degree of conduction slowing appears to be variable, with some studies demonstrating no slowing and others reporting 23%–44% reduced conduction velocity (reviewed in Tse and Yeo, 2015). This apparent disparity has been linked to the ionic composition of the extracellular fluid and ephaptic coupling (George and Poelzing, 2016). In line with the findings from Cx43 knockdown studies, slowing of the conduction velocity from 0.23 to 0.16 m/s by means of a low concentration of heptanol (0.05 mM) was found to be sufficient to increase the inducibility of ventricular tachycardia in isolated mouse hearts. This occurred without affecting the effective refractory period, so that cardiac excitation wavelength (the product of conduction velocity and effective refractory period) was shortened (Tse et al., 2016b).

For propagation along the bundle of His, the Tawara branches, and the Purkinje fibers, it is important that the conduction bundle does not communicate with the surrounding cardiac tissue, because otherwise the activation would start at the valvular level. This isolation is achieved by differential connexin expression: the conduction system communicates mainly via

Cx40-containing GJ channels, whereas the working myocardium communicates via Cx43 channels. A specific biophysical problem occurs at the interface between these systems at the endings of the Purkinje fibers where the small Purkinje fiber has to activate a large surrounding tissue (known as the "current source/sink problem"). Elicitation of an action potential requires a depolarizing current from an adjacent, already activated cell. The activated cell represents a current source and the quiescent cell a current sink. The voltage difference between these two cells is the driving force for this current that flows via GJ channels (and, to some extent, via the extracellular space). The quantity of current transferred is determined by geometric properties of the tissue. Thus, if a tiny cluster of activated cardiomyocytes (i.e., a small source) needs to activate a large surrounding region (a large sink), current flows radially from the activated cluster to many nonactivated sites, so that the current is distributed to a large number of cells. As a consequence, the total charge transferred to each of these neighboring cells may be too low to activate these cells, thereby causing conduction failure (Rohr et al., 1997; Rohr, 2004, 2012; Lee and Pogwizd, 2006). If GJ conductance is reduced, it may prevent conduction block, since less current is lost to the adjacent cells. Thus, a small source can activate only a very limited number of neighboring cells. A similar situation is present in the sinus node, where the weak conduction between a tiny current source (sinus node) and a large sink (atrium) is achieved by a limited expression of GJs localized in in interdigitating finger-like zones extending from the sinus node into the atrium (Joyner and van Capelle, 1986; Boyett et al., 2006). In hypokalemia, shortening of ventricular effective refractory period without changes in conduction velocity has been observed. Interestingly 0.1 mM heptanol restored the effective refractory period and acted anti-arrhythmically, which may support the hypothesis that also GJ reduction may prevent from arrhythmia in certain situations (Tse et al., 2016a).

A general mathematical formulation describes the ratio of charge produced/charge consumed as the safety factor, which equals $Q_c + Q_{out}/Q_{in}$ (Shaw and Rudy, 1997a,b). If coupling is reduced gradually, the safety factor first is enhanced as a result of the smaller space constant. However, if very low levels of coupling are reached, SF decreases until $SF < 1$ and conduction failure occurs (Shaw and Rudy, 1997a,b). A high curvature of the propagation wave front typically causes such source-sink-problems. Moreover, typical situations with source-sink problems are the endings of Purkinje fibers, areas in which the activation front passes a narrow isthmus, encircles obstacles, or at the border between ischemic and nonischemic tissue, where the ischemic nonexcitable tissue acts as a large current sink. The relationship between cell surface (A_m, which is linearly related to cell capacitance) and GJ conductance (g_{GJ}) is also important. An increased cell diameter may accelerate longitudinal propagation velocity θ_L, if g_{GJ} is enhanced proportionally to the cellular membrane surface A_m. In contrast, in the case of constant g_{GJ}, increased diameter leads to reduced θ_L. Consequently, the g_{GJ}/A_m ratio controls whether alterations in cell size will enhance or reduce θ_L (Seidel et al., 2010).

Taken together, altered arrhythmogenicity in relation to GJs may be explained by alterations in conduction velocity, in particular conduction failure either due to complete block of conduction or to a source-sink mismatch, by alterations in conduction pathways, by changes in refractory periods, e.g., due to enhanced or reduced current flow via GJs to adjacent cells. A comprehensive overview of the biophysical basis of arrhythmia and normal conduction is presented in Dhein et al. (2014). Below we further discuss the role of GJs in ventricular and atrial fibrillation.

A. Gap Junctions and Ventricular Fibrillation

Ventricular fibrillation (VF) is among the most dangerous arrhythmias. It often occurs after acute or chronic myocardial infarction or in patients with heart failure. Typically VF is driven by a spiral wave rotator that encircles a nonexcitable obstacle like a scar (Krinsky, 1978) or a permanently depolarized area (Karagueuzian et al., 1998), forming an abnormal circuit like the "leading circle" identified in the atrium (Allessie et al., 1977). According to these theories, ventricular fibrillation is driven either by a single rapidly drifting rotor or by a stationary rotor (Samie and Jalife, 2001). Such spiral waves are promoted by reduced excitability or reduced conduction velocity (Mandapati et al., 1998). The diameter of a rotator may be very small (hundreds of micrometers). In infarcted hearts, the infarct zone often is not a homogenous zone, but contains patchy fibrosis; the likelihood of VF induction is positively related to the patchiness of this area (Karagueuzian et al., 1986).

Structurally, VF is favored by nonuniform anisotropy, e.g., small islets of connective tissue, fibroblasts, or scars. These structures lead to 1) a delay in conduction (Rohr et al., 1997) and 2) inhomogeneity in action potential duration (APD) (Lesh et al., 1989; Müller and Dhein, 1993) resulting in shortened APD at sites where a cell encounters a large nonactivated area (current sink) and where conduction delay may prolong the time during which activated cells lose current to adjacent sites (Müller and Dhein, 1993; Spach and Josephson, 1994; Gottwald et al., 1998). The interaction of cardiomyocytes and nonexcitable fibroblasts in such areas is a topic of substantial ongoing investigation. Both of these cell types can be coupled via GJs typically consisting of Cx45 or Cx43 as determined from immunostainings or from their single channel conductances (Rook et al., 1989; Camelliti et al., 2004), although the

fibroblast is electrically inactive. Such heterocellular communication may explain the conduction of action potentials for up to 300 μm through connective tissue/fibroblast areas (Rohr, 2004; Brown et al., 2015), but with significantly slowed conduction velocity (Nguyen et al., 2014). In aged hearts, the degree of fibrosis is enhanced, and as a consequence, the activation pattern shows more irregularities (Dhein and Hammerath, 2001). Similarly, enhanced microfibrosis is also found in failing hearts (Glukhov et al., 2012). Stem cells seem to be able to establish GJ-mediated intercellular communication with cardiomyocytes and may similarly generate a risk for arrhythmia (Smit and Coronel, 2014). Most recently it was reported that even macrophages can form Cx43-based GJs with cardiomyocytes, thereby assisting in AV nodal conduction but also affecting repolarization (Hulsmans et al., 2017).

What is the role of GJs in conditions leading to ventricular fibrillation? In acute ischemia (see Fig. 4), intracellular pH drops and ATP is lost; consequently, the activity of membrane pumps like the Na^+/K^+ pump and the Na^+/Ca^{2+} exchanger are reduced, resulting in increased intracellular Na^+ and Ca^{2+} concentrations and membrane depolarization. ATP loss and accumulation of Na^+, H^+, and Ca^{2+} inside the cells reduce GJ conductance. Intracellular acidosis activates the Na^+/H^+ exchanger and the HCO_3^-/Na^+ symporter, thereby enhancing the intracellular rise in Na^+. In consequence of reduced GJ conductance and Na^+ current availability, conduction is delayed or even blocked. In addition, lysophosphoglycerides, long chain acylcarnitines, and arachidonic acid metabolites accumulate and further reduce GJ intercellular communication (Wu et al., 1993). More recent investigations also found that myocardial Cx43 becomes dephosphorylated after 30 minutes of regional ischemia and is removed from the cell membrane accompanied by conduction slowing (Jozwiak and Dhein, 2008). Conduction slowing may allow the activation wave to circle around a nonexcitable zone, reaching its starting point when repolarization has just finished. This condition is a typical condition to establish for reentry circuits.

In acute ischemia, there are two peaks of VF incidence (Smith et al., 1995): the first (type Ia) occurs within 5 minutes after onset of ischemia, the second (type Ib) occurs after about 20–30 minutes. The early peak is often related to factors such as catecholamine release, depolarization, and mechanical factors together with shortened action potentials. The second peak (Ib) has been linked to GJ uncoupling, since increased tissue impedance and conduction slowing were observed after 12–15 minute of ischemia (Dekker et al., 1996; Müller et al., 1997b; de Groot et al., 2001). This uncoupling is caused by ATP depletion, intracellular acidosis, accumulation of acylcarnitines, dephosphorylation of Cx43 (especially at Ser-368) (Lampe et al., 2006; Jozwiak and Dhein, 2008), intracellular Na^+ and Ca^{2+} overload due failure of the Na^+/K^+-ATPase, and Na^+/Ca^{2+} exchanger (Burt, 1987; Fluri et al., 1990; Massey et al., 1992; Morley et al., 1996; Hofgaard et al., 2008). Such ischemic uncoupling can be antagonized by antiarrhythmic peptides like AAP10 (Müller et al., 1997b; Dhein et al., 1994; Jozwiak and Dhein, 2008) or its derivative rotigaptide (Kjølbye et al., 2003; Xing et al., 2003). AAP10 has been shown to maintain the degree of phosphorylation of Cx43 in a PKC-dependent manner and to preserve the localization of Cx43 at the cell poles (Jozwiak and Dhein, 2008). In contrast, ischemia leads to a reduction in membrane-associated Cx43 and to a loss of polar Cx43. The inducibility of ventricular fibrillation may be regulated by a domain within the Cx43 CT region (Maass et al., 2009).

Ischemic uncoupling is typically accompanied by depolarization. Reduced I(Na) availability enhances dispersion of the action potential duration (APD), since local differences in APD causing voltage gradients can no longer be neutralized by GJ currents. Dispersion and conduction slowing (that occur in some cases) and nonexcitable areas (e.g., depolarized areas) are factors known to initiate reentrant arrhythmias (Janse, 1986; Janse et al., 1986; Coronel et al., 1989; Opthof et al., 1991; Wit and Janse, 1992; Spach and Josephson, 1994; el-Sherif et al., 1996). The voltage gradients between ischemic and nonischemic areas may also generate a flow of injury current that may be strong enough to depolarize surviving Purkinje strands (Janse et al., 1980).

Chronic infarction is characterized by necrosis of cardiomyocytes and replacement by connective tissue; it may produce electrically silent zones that interdigitate among normal cardiac tissue. It produces local inhomogeneity and conduction, slowing, or even conduction failure. The loss of contractile force reduces ejection fraction, causing left ventricular dilatation and increased wall tension with mechanical stretch.

As introduced in *section V*, Cx43 expression is reduced in chronic infarction (Peters, 1995; Chen et al., 2010), resulting in reduced GJ communication and consequent conduction slowing (Yao et al., 2003). Some investigators have also observed increased abundance of Cx43 at the lateral side of the cardiomyocytes (Kieken et al., 2009), which (if functional) may result in enhanced transverse conduction and reduced anisotropy. The increased Cx43 expression may be regulated by angiotensin II, endothelin, or enhanced mechanical stretch (especially if cardiac function is compromised and cells become hypertrophic). Interactions between cardiomyocytes and fibroblasts may also be important in chronic infarction. Cocultures of both cell types (separated by a semipermeable membrane) revealed that fibroblasts release a soluble factor that inhibits β-adrenoreceptor-dependent increases in Cx43 expression (Salameh et al., 2013). If the infarcted area exceeds a certain size, heart

failure occurs (perhaps associated with ischemic cardiomyopathy, see below).

Alterations in Cx43 expression and GJs are also found in heart failure, potentially contributing to associated arrhythmias. Heart failure may be characterized as compensated heart failure, hypertrophy, and decompensated heart failure and classified by four levels (NYHA I–IV), with the most severe level (IV) corresponding to patients who suffer from dyspnea even when lying in bed. The typical therapeutic option for NYHA IV patients is cardiac transplantation. "Heart failure specimens" for many studies derive from the explanted hearts of these patients; studies of gene and protein expression and regulation may be confounded by the many drugs that are given to these patients. Heart failure may also be distinguished according to pathophysiological considerations including 1) ischemic cardiomyopathy (ICM), 2) dilated cardiomyopathy (DCM), and 3) hypertrophic cardiomyopathy. Salameh et al. (2009) showed that polar and lateral Cx43 are increased in hypertrophic cardiomyopathy, but the polar fraction is reduced and the lateral fraction is nearly unchanged in cardiac biopsies from patients with dilated cardiomyopathy (NYHA II–III) (Salameh et al., 2009). These authors observed increased Cx43mRNA and protein expression in hypertrophic cardiomyopathy patients and decreased Cx43mRNA and Cx43 protein in DCM patients. The diminished Cx43 levels observed in DCM patients are accompanied by downregulated ZO-1 protein (Kostin, 2007). ZO-1 is a scaffolding protein for Cx43, and reductions of Cx43 may be result from its degradation after displacement from ZO-1 after phosphorylation by c-Src (Sovari et al., 2011; Rutledge et al., 2014). The regulators of altered Cx43 expression in heart failure include angiotensin II, endothelin, bFGF, catecholamines, and TNF-α (reviewed by Dhein, 2004).

In many cases, the pathogenic alterations of GJs may not be uniform across the ventricle. Reduced Cx43 expression was observed in samples obtained from human patients suffering from ICM, DCM, or inflammatory cardiomyopathies; but, interestingly, especially in the ICM patient samples, both areas with clearly reduced Cx43 expression and areas with nearly normal Cx43 expression and distribution were identified (Kostin et al., 2003). Thus, Kostin et al. (2003) concluded that "focal disorganization of GJ distribution and downregulation of Cx43 are typical features of myocardial remodeling that may play an important role in the development of an arrhythmogenic substrate in human cardiomyopathies."

It is apparent that local disorganizations of ventricular GJs may be more important for cardiac pathophysiology than overall changes in expression (Kitamura et al., 2002). Such a focal disorganization in the communicating GJ network together with fibrosis and changes in the cellular geometry (due to hypertrophic processes) may cause effects on the current source-sink relationships that can dramatically affect overall conduction and local propagation patterns (Spach and Dolber, 1990; Rohr et al., 1997; Spach et al., 2000; Rohr, 2004, 2012; Lee and Pogwizd, 2006; Seidel et al., 2010). Thus, the network consisting of cardiomyocytes with their specific shape, irregularities in Cx43 expression, changes in subcellular Cx43 distribution, microfibrosis, nonexcitable cells (like fibroblasts) can form the arrhythmogenic substrate.

Two factors affecting conduction are consistently observed in chronic heart disease: 1) the degree of fibrosis is enhanced, which increases nonuniformity, and 2) the expression/distribution of GJ proteins is altered. Often the abundance of connexins at the lateral side of the cardiomyocytes is increased relative to that at the ends. Increased coupling by these lateral junctions could increase transverse conduction (Seidel et al., 2010). Hypertrophy associated changes in cell size and geometry will also alter anisotropy. Altered ratios between longitudinal and transverse conduction have been described in ventricular preparations, atrial tissue, and computer simulations (Spach et al., 2000; Koura et al., 2002; Hubbard et al., 2007) (Dhein and Hammerath, 2001; Seidel et al., 2010; Dhein et al., 2011). These effects may be modulated by alterations of the extracellular space (Cabo and Boyden, 2009).

B. Gap Junctions and Atrial Fibrillation

Atrial fibrillation (AF) is the most common cardiac arrhythmia. It is characterized by a rapid and irregular electrical activation and the loss of atrial muscle contractility. The pathogenesis of AF involves initiating triggers (often rapidly firing ectopic foci located inside the pulmonary veins) and an abnormal atrial tissue substrate that maintains the arrhythmia (Allessie et al., 2001; Iwasaki et al., 2011). The tissue substrate is determined in large part by the abundance and distribution of intercellular channels contained within atrial GJs (Spach and Starmer, 1995).

Atrial GJs are comprised of two different subunit proteins, Cx40 and Cx43. They are each abundantly expressed by atrial myocytes with similar abundances and highly overlapping distributions (Lin et al., 2010; Gemel et al., 2014a). Together, Cx40 and Cx43 determine the properties of intercellular conduction within this tissue (Saffitz et al., 1994; Lin et al., 2010). A number of studies have investigated the possible role for GJs and connexins in atrial fibrillation in animal models or in human patients (reviewed by Dhein, 2006b; Duffy and Wit, 2008). Likely reflecting the heterogeneity of this disorder (idiopathic versus secondary and paroxysymal versus persistent versus permanent), a wide diversity of different results have been obtained including increases, decreases, or lack of changes. Various alterations of both Cx40 and Cx43 have been observed in animals and human patients with AF (reviewed in Kato et al., 2012). Below, we

further discuss alterations of these connexins at the level of messenger RNA, protein expression, posttranslational modifications, distribution, and genetic abnormalities or mutations.

1. Connexin mRNAs. Taken together, there is little consistent evidence to suggest significant changes in Cx43 expression or levels. Cx40 mRNA levels are reduced in some mice with cardiac targeted defects in the heart (Sawaya et al., 2006), but other animal studies provide little evidence for alterations of Cx40 mRNA (van der Velden et al., 2000). Cx40 mRNA may be reduced in some patients with valvular heart disease or heart failure associated with AF (Nao et al., 2003; Gaborit et al., 2005), but other studies have shown no changes. Moreover, examination of the expression profile data available in the GEO data sets (http://www.ncbi.nlm.nih.gov/projects/geo/gds) shows no consistent difference in Cx40 mRNA (or that of other connexins) between AF and control patients.

2. Connexin Protein Levels. Most reports have observed no major changes in Cx43 abundance detectable by immunoblotting (Polontchouk et al., 2001). Perhaps because good anti-Cx40 reagents had not been widely available until recently, there is little published data regarding Cx40 levels detected by immunoblotting. Immunohistochemistry has been used in a number of studies to estimate levels of immunoreactive Cx40 or Cx43. Some studies have found decreases in Cx40 and/or Cx43 (Kostin et al., 2002), but the most consistent finding is a decrease in the Cx40/Cx43 ratio (implying a greater reduction of Cx40 than Cx43) (van der Velden et al., 2000; Kanagaratnam et al., 2004; Wilhelm et al., 2006). Since mediators like angiotensin II (which can be released in AF or upon stretch) can upregulate Cx43 expression and other factors like ischemia can reduce Cx43 expression, the degree and the type of alteration of connexin expression (increase or decrease) is known to be affected by the comorbidity of the patient (see e.g., Wetzel et al., 2005). Thus it is necessary to differentiate at least between lone AF, mitral valve disease related AF, and AF in coronary heart disease.

3. Posttranslational Modifications of Connexins. As noted above, the major cardiac connexins are all phosphoproteins, and different phosphorylation events can regulate many aspects of GJ assembly, function, and degradation. c-Jun N-terminal kinase (JNK) activation is implicated in cardiovascular diseases and aging, which are linked to enhanced propensity to atrial fibrillation (AF). In a rabbit model, Yan et al. (2013) showed that JNK activation contributed to Cx43 reductions that promote development of AF (Yan et al., 2013).

4. Connexin Distribution. The other frequent finding in both animal models and human AF is heterogeneity of connexin staining (i.e., some areas show intense GJ immunostaining, whereas others show weak or no staining). When both connexins have been studied in the same samples, the heterogeneity is often more severe for Cx40 (van der Velden et al., 1998; Kanagaratnam et al., 2004).

5. Genetic Abnormalities of Cx40 Linked to Atrial Fibrillation. Two groups of studies suggest AF-associated genetic abnormalities of Cx40. The Cx40 (GJA5) gene contains two closely linked polymorphisms in the promoter region, −44 (G→A) and +71 (A→G). One haplotype (−44A/+71G) is relatively rare, and it has been associated with familial atrial standstill (Groenewegen et al., 2003) and atrial fibrillation (Hauer et al., 2006). This polymorphic variant has reduced promoter activity in in vitro reporter gene experiments (Firouzi et al., 2006), suggesting that it might lead to reduced Cx40 expression in people who carry this polymorphism. Coding region variants of Cx40 may also sometimes contribute to AF. Gollob et al. (2006) examined the sequence of the Cx40 coding region in atrial tissue obtained from 15 patients with lone AF (Gollob et al., 2006). They identified four patients with mutations: three patients apparently had somatic mutations and the fourth patient had a germline mutation. Subsequent reports have identified individuals or families with additional germline mutations (Yang et al., 2010). Expression studies have shown that some of these mutants have defects like impaired GJ formation, accelerated degradation, or abnormal GJ or HC function that might contribute to AF pathophysiology (Gollob et al., 2006; Sun et al., 2013; Patel et al., 2014; Gemel et al., 2014b).

6. Cx43 Mutation Linked to Atrial Fibrillation. Cx43 mutations may also be a genetic cause of AF. A single nucleotide deletion (c.932.delC) of the Cx43 encoding gene (GJA1) was identified by Thibodeau et al. (2010) in DNA prepared from the atrial tissue of a patient with AF (Thibodeau et al., 2010). The mutation resulted in a frame shift, premature truncation, and a Cx43 polypeptide with impaired cellular trafficking that acted as a dominant negative inhibitor of coexpressed wild-type Cx43 or Cx40. Absence of the mutation in patient leukocyte DNA suggested genetic mosaicism.

In most cases, AF is a secondary event resulting from other cardiac abnormalities like chronic heart failure or valvular disease. In such patients, secondary alterations of Cx40/Cx43 levels and distributions may contribute to the pathophysiology of AF. About 15% of AF patients have "lone AF," which develops in apparently normal hearts in the absence of structural abnormalities. It appears that mutations of Cx40 only rarely cause AF in these patients since they have not been identified very often (Tchou et al., 2012; Gemel et al., 2014a). In contrast, a study of atrial tissue obtained from a group of patients who underwent surgical ablation of their lone AF by Gemel et al. (2014) showed that that reduced Cx40 levels and heterogeneity of its

distribution (relative to Cx43) were common in AF (Gemel et al., 2014a).

C. Further Genetic Considerations Regarding Gap Junctions and Arrhythmia

Studies in genetically manipulated mice implicated Cx40 in various arrhythmias. The initial descriptions of Cx40-deficient mice showed that they had cardiac conduction abnormalities including atrioventricular and bundle branch block (Simon et al., 1998) or reduced cardiac conduction velocity and predisposition to atrial arrhythmias (Kirchhoff et al., 1998). Subsequent studies confirmed roles for Cx40 in the specialized conduction system, since $Cx40^{-/-}$ mouse hearts exhibit right bundle-branch block, delayed atrioventricular nodal and proximal His bundle conduction, and impaired left bundle conduction (Tamaddon et al., 2000; van Rijen et al., 2001) and in the atrium based on findings of long sinus node recovery time, sinus entry block, slow atrial conduction, and atrial tachyarrhythmias (Kirchhoff et al., 1998; Hagendorff et al., 1999; Verheule et al., 1999). After atrial burst pacing, $Cx40^{-/-}$ mice develop atrial tachyarrhythmias (Bagwe et al., 2005).

Genetically manipulated mice have also provided convincing evidence for the importance of Cx43 in ventricular arrhythmias. Gutstein et al. (2001a) generated mice with cardiac-specific loss of Cx43 (homozygous Cx43 conditional KO mice with mosaicism, giving 86%–95% decreased expression at 4 weeks age) (Gutstein et al., 2001a). Despite normal heart structure and contractile function, these mice had reduced ventricular conduction velocities and uniformly developed sudden cardiac death from spontaneous ventricular arrhythmias.

However, besides the AF-associated connexin mutations discussed above, there are only a few additional reports linking cardiac connexin mutations to arrhythmias. Makita et al. (2012) identified a Cx40 (GJA5) mutation (encoding the substitution Q58L) in affected members of a family with Progressive Familial Heart Block Type I, a dominant inherited disorder of the His-Purkinje system causing heart block (Makita et al., 2012). Van Norstrand et al. (2012a) identified two missense mutations of Cx43 (E42K and S272P) among a series of cases of Sudden Infant Death Syndrome (Van Norstrand et al., 2012a).

In contrast, a large number of people carry Cx43 mutations without any apparent cardiac electrical phenotype. Cx43 mutations have been identified as the cause of ODDD, an autosomal dominant syndrome that is primarily characterized by craniofacial and limb abnormalities (Paznekas et al., 2003, 2009). Although a large number of different Cx43 mutations have been identified in ODDD patients (78 to date), cardiac arrhythmias are strikingly absent except for the I130T mutant that has been linked to ventricular tachyarrhythmia (see *section VII*).

Connexin-related arrhythmias may result from other genetic diseases. Patients with arrhythmogenic cardiomyopathy have an inherited disease characterized by fibro-fatty replacement of the myocardium (especially in the right ventricle) and severe ventricular arrhythmias. The familial form of the disease is linked to mutations of desmosomal proteins (Saffitz, 2011). The arrhythmias may result from loss of GJ plaques as observed in the ventricular tissue of these patients (Kaplan et al., 2004), which, in turn, may result from absent interactions of Cx43 with desmosomal components (Oxford et al., 2007).

We conclude that myocardial GJs and their component connexins (especially Cx40 and Cx43) are critical for normal cardiac conduction. Their perturbations can contribute to arrhythmias. Rarely, there may be a specific genetic abnormality of the connexin, but, most commonly, the pathogenic changes of the connexins are local and heterogeneous alterations of distribution and abundance that are secondary to other cardiac pathologies.

VII. Cx43 in Oculodentodigital Dysplasia

Many GJ studies from the eighties were focused on the isolation of the protein that was responsible for junctional intercellular communication in the heart. By using a clever detergent-resistant approach, microgram quantities of pure GJs were obtained from dozens of pooled rat hearts (Manjunath and Page, 1985; Manjunath et al., 1987). Upon examination of the protein content of these purified GJs, a protein of 43 kDa in molecular mass was discovered that appeared as multiple bands that were later defined as phosphorylation species of the cardiac GJ protein (Yancey et al., 1989; Crow et al., 1990; Laird and Revel, 1990; Musil et al., 1990; Laird et al., 1991). In 1987, Beyer et al. (1987) would go on to clone the major cardiac GJ protein and define it as Cx43. Given the abundance of Cx43 in the ventricles, Cx43 was projected to be absolutely critical for the synchronous beating of the ventricular cardiomyocytes and that any attenuation of its expression or function would quickly lead to death. In 1995, when the Kidder and Rossant laboratories reported the first Cx43 knockout mouse, it was not too surprising that these mice died shortly after birth due to swelling and blockage of the right ventricle outflow tract that caused a failure in pulmonary gas exchange (Reaume et al., 1995). What was probably more surprising was that mice heterozygous for *Gja1* gene ablation had no overt cardiac malformation or functional limitations and would go on to live a relatively normal life span (Reaume et al., 1995). Thus, mice could function well on half the normal Cx43 content in the heart as mentioned earlier (see *section VI*). These findings began to challenge the paradigm that had emerged over the previous years that an exquisite and tight regulation of Cx43 in

the mouse heart was necessary to prevent developmental disorders and cardiac disease. At this time in history, the GJ community was only beginning to become aware of the fact that germline mutations in connexin encoding genes could cause disease. Upon the publication of *GJB1* gene mutations linked to X-linked Charcot-Marie-Tooth disease (Cochrane et al., 1994), the search was on to see whether humans with *GJA1* gene mutations existed in society as many predicted that such mutations would be embryonically lethal and no fetuses would survive through to birth.

A. The Discovery of the Genetic Basis of Oculodentodigital Dysplasia

In a seminal paper from Ethylin Jabs's laboratory in 2003, they reported the first 17 families that harbored *GJA1* gene mutations found in a patient group that was defined as suffering from a rare developmental disorder known as ODDD (Paznekas et al., 2003). Clinically, this disease had been well documented for over several decades, but the genomic cause of this condition had remained elusive (Gorlin et al., 1963; Eidelman et al., 1967). ODDD is a complex disease as no two patients seem to present with identical disease (Paznekas et al., 2009). Many patients exhibit small eyes, a loss of tooth enamel, fusion of the digits, and craniofacial defects linked to both bone and cartilage (Paznekas et al., 2009). However, at the individual level, patients may be clinically quite diverse and suffer from upwards of a dozen other conditions that include incontinence, skin diseases, thermosensitive skin, glaucoma, and many other syndromes (Paznekas et al., 2009). Importantly, comorbidities that often accompany more classic characteristics of ODDD, like decreased mobility, increase with age and appear to be linked to deteriorations in the central nervous system (De Bock et al., 2013a). Given the ubiquitous distribution of Cx43 in most human organs, it was equally intriguing to discover what organs were unaffected by *GJA1* mutations (Laird, 2014). To that end, the development of the heart and cardiac function is seldom casually affected by a patient harboring a *GJA1* gene mutation (Paznekas et al., 2009; Laird, 2014). Over 135 papers have now been published that present the breadth of clinical presentations found

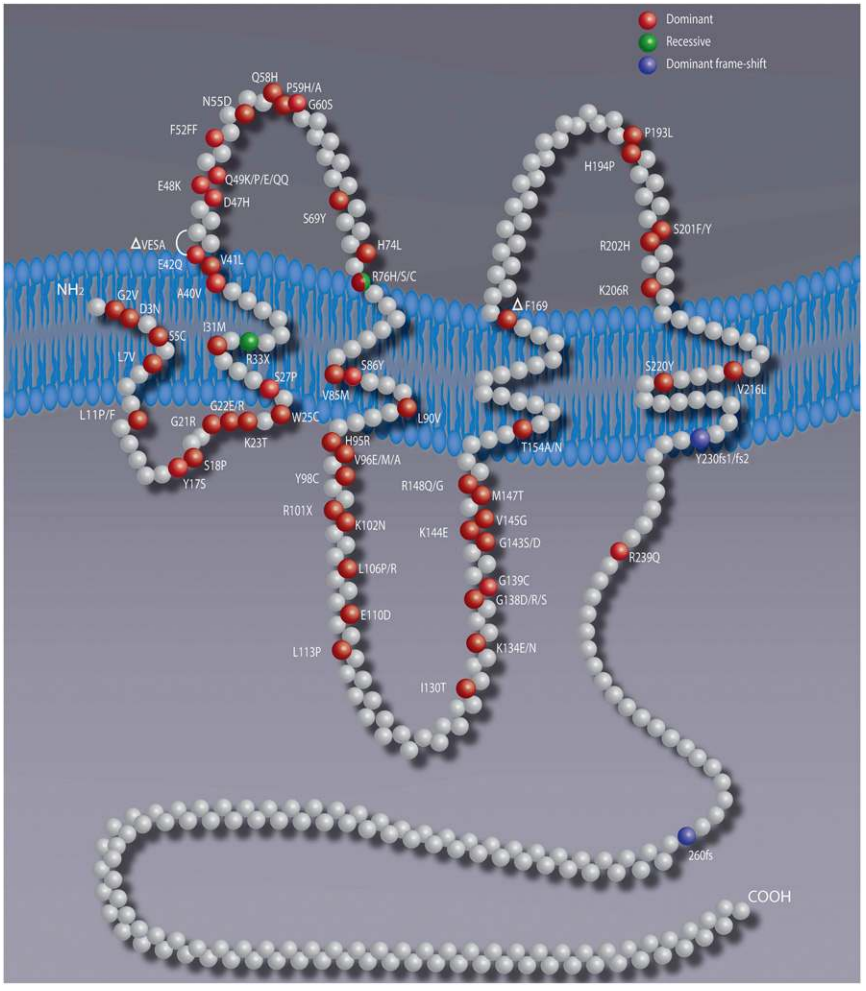

Fig. 8. Composite map of the Cx43 polypeptide depicting all the known *GJA1* gene mutations linked to ODDD.

in patients, the *GJA1* mutations that cause ODDD, and the molecular mechanisms that are at the root of the disease.

B. GJA1 Gene Mutations Linked to Oculodentodigital Dysplasia and Beyond

At last count there are 78 mutations in the *GJA1* gene linked to ODDD (Fig. 8) (Laird, 2014). Over 90% of these mutations are autosomal dominant that mostly result in single amino acid substitutions (Fig. 8). On the first level of interrogation, one might predict that these amino acid substitutions would be substantial changing the charge, hydrophobicity, hydrophilicity, or secondary structure of the motif or domain where they reside. Although that is the case in some instances, it is equally probable that the substituted amino acid is conservative in nature, which is predicted to have minimal effects on the secondary or tertiary structure of Cx43. The second most intriguing observation when surveying the motifs and domains that harbor Cx43 mutations is the fact that they are almost entirely located within the first two-thirds of the Cx43 polypeptide sequence (Fig. 8). This observation indirectly suggests that mutations in the *GJA1* gene that encode the C-terminal domain of Cx43 are probably embryonically lethal. This notion is not entirely true, because three autosomal dominant frame-shift mutations have been identified at residues 230 (two different mutations) and 260, suggesting that human survival is indeed possible if the C terminus is ablated from one allele, although very few patients worldwide have been identified with this class of mutations (Laird, 2014). Two autosomal recessive mutations have also been identified and linked to ODDD, one mutation truncates Cx43 at residue 33 and a second mutation causes an amino acid substitution at residue 76 (Paznekas et al., 2009; Laird, 2014). In the case of the homozygous mutation that leads to a severe truncation of Cx43 at residue 33, which is the equivalent to a Cx43 knockout in humans, these young children were severely ill and exhibited multisystem functional disorders (Paznekas et al., 2009).

Although 100% of ODDD patients genotyped harbor *GJA1* gene mutations, there are a few cases of *GJA1* gene mutations that do not cause ODDD. For example, the autosomal recessively inherited R239Q mutant has been shown to cause a related syndrome to ODDD called craniometaphyseal dysplasia (Hu et al., 2013). Two other mutants (E42K and S272P) have been linked to sudden infant death by mechanisms that remain unclear, inasmuch as the E42K mutant has perturbed Cx43 properties while the S272P appears to function normally (Van Norstrand et al., 2012b; Lübkemeier et al., 2015). Still other mutations as found in patients homozygous for the R76H amino acid substitution exhibit ODDD but have also been classified to present with Hallermann-Streiff syndrome characterized by a small stature, congenital cataracts, hypotrichosis, beaked nose, skeletal anomalies, and teeth defects (Pizzuti et al., 2004). Thus, some caution must be exercised in concluding that all mutations in the *GJA1* gene will result in patients presenting with ODDD or ODDD only.

C. Molecular Mechanisms Linking GJA1 Gene Mutations to Oculodentodigital Dysplasia

Over two dozen ODDD-linked Cx43 mutants have been engineered and interrogated when expressed in reference cells or tissue-relevant cellular environments (De Bock et al., 2013a; Laird, 2014). In some cases, these host cells coexpressed, or were engineered to express, Cx43 in an attempt to mimic the autosomal dominant mode of disease inheritance, or the host cells expressed other members of the large connexin family of 21 genes (De Bock et al., 2013a; Laird, 2014). In all ectopic mutant-expressing situations, it is challenging to create physiological relevant ratios of Cx43 to disease-linked Cx43 mutants and also to have appropriate levels of Cx43 mutants coexpressed with native levels of other connexin family members. Nevertheless, these strategies were successfully used to determine that the mechanisms responsible for Cx43 mutants causing disease are highly variable and can be first classified into either loss-of-function or gain-of-function mutants (Shibayama et al., 2005; Laird, 2006, 2008, 2014; De Bock et al., 2013a). Loss-of-function mutations are found throughout much of the polypeptide sequence and include many amino acid substitutions within the amino terminal of Cx43 where the resulting mutants could traffic to the cell surface and assemble into GJ-like structures, but these channels were not functional (e.g., G21R) (Roscoe et al., 2005; Gong et al., 2007; Shao et al., 2012). Other loss-of-function mutants included frame-shift mutations that caused the C terminus of Cx43 to be lost, and these mutants were retained within the endoplasmic reticulum or Golgi apparatus because they failed the quality control assessment to be able to traffic to the cell surface (e.g., fs230, fs260) (Gong et al., 2006; Churko et al., 2010). Still other loss-of-function mutants assembled into channels, but the resulting channels, both GJ and HCs, were not fully functional, suggesting changes in channel pore or gating mechanisms (e.g., Y17S, G21R, A40V, L90V, and I130T illustrated in Fig. 1) (Lai et al., 2006; Kalcheva et al., 2007; Churko et al., 2010; Lorentz et al., 2012; De Bock et al., 2013a; Stewart et al., 2013; Huang et al., 2014). Other loss-of-function ODDD-linked mutants cannot be assigned to one category and exhibited combinatory defects in trafficking, assembly, and/or function.

Somewhat less expected, ODDD-linked mutants acquired new or enhanced properties that can be best described as gain-of-function mutants. For example, several ODDD-linked mutants (I31M, G60S, G138R, and G143S illustrated in Fig. 1) exhibited a tendency to

acquire an open HC state or form leaky HCs, suggesting that the tight regulation of maintaining HCs closed at the cell surface was dysfunctional; interestingly this gain-of-function of HCs was associated with decreased function of GJs (Dobrowolski et al., 2007; Dobrowolski et al., 2008; Kozoriz et al., 2013). Still other ODDD mutants may acquire the ability to interact with other coexpressed connexin family members. Concrete examples of this are difficult to pinpoint in ODDD but are more commonly found in Cx26-linked skin diseases where Cx26 mutants can surprisingly acquire the ability to impair the function of Cx43, a connexin not typically able to bind Cx26 (Rouan et al., 2001; Thomas et al., 2004). Since Cx43 is thought to interact with Cx40 and Cx45 (Laing et al., 2001; Bouvier et al., 2009), when considering the heart, one might propose that Cx43 mutants may lose the ability to bind Cx40 or Cx45, thus allowing these cardiac connexins to rescue the heart from disease in ODDD patients. Other gain-of-function mechanisms where mutants acquire the ability to bind novel proteins have been proposed, but definitive examples of this mechanisms are not well established.

D. Insights from Mouse Models

Given that ODDD is typically an autosomal dominant disease, the need emerged to generate genetically modified mice that were heterozygous for the Cx43 gene mutation and thus mimic the human condition. Through the use of an *N*-ethyl-*N*-nitrosurea mutagenesis screen, in 2005 Janet Rossant and team identified a Cx43 G60S mutant mouse that mimicked human ODDD (Flenniken et al., 2005). Although this particular mutant has never been found in the human population, the mutant mouse mirrored the human disease as revealed by fusion of the digits, loss of tooth enamel, small eyes, and craniofacial bone defects (Flenniken et al., 2005). This mouse has been used as a surrogate of human ODDD in nearly two dozen publications that have served to uncover how a loss-of-function Cx43 mutant causes disease or defects in bone, teeth, reproductive organs, bladder, eyes, and the CNS while sparing other Cx43-rich tissues like the heart and vessels (Toth et al., 2010; Tsui et al., 2011; Lorentz et al., 2012; Kozoriz et al., 2013; Stewart et al., 2013; Winterhager et al., 2013; Zappitelli et al., 2015).

In addition to ODDD patients presenting with multisystem syndromes, it was noted that a few Cx43 gene mutations appeared possibly to predispose patients to potential cardiac defects (Paznekas et al., 2003, 2009). One of these mutations was I130T, which was engineered into a mouse model of ODDD to investigate if this particular Cx43 mutant would lead to notable cardiac dysfunctions including arrhythmias (Kalcheva et al., 2007). Here the authors found that these mice did have significantly less Cx43 in the functional myocardium that corresponded with less functional coupling and slowing of conduction velocity (Kalcheva et al., 2007). Furthermore, these mice did exhibit increased susceptibility to ventricular tachyarrhythmias (Kalcheva et al., 2007). Unfortunately, there is not a sufficient number of patients that harbor the I130T mutation to determine if these clinical presentations are consistently present in humans that harbor this mutation. However, these studies open up the possibility that one or more ODDD-linked mutants may in fact cause a cardiac phenotype.

A third mouse that mimics ODDD was generated by conditionally knocking in the Cx43 G138R mutant, which allows for both unaffected and affected organs to be examined in a tissue-specific manner (Dobrowolski et al., 2008). By using this strategy, mice that selectively expressed the mutant in cardiomyocytes developed spontaneous arrhythmias that were found to correlate with a reduction in Cx43 GJ function (Dobrowolski et al., 2008). In this case, the authors concluded that changes in rhythmogenesis were due to increases in ATP release through Cx43 HCs that had become leaky due to the mutation. Here, the proposed gain-of-function induced by the G138R mutant appeared to be responsible for the ODDD phenotype (Dobrowolski et al., 2007). In another study, mice conditionally expressing the G138R mutant in the limb bud developed syndactylies due to impaired apoptosis, a condition linked to reduced expression of sonic hedgehog and bone morphogenic protein 2 (Dobrowolski et al., 2009). These later studies point to a possible second mechanism of how the G138R mutant can lead to tissue changes that present in ODDD-linked phenotypes. In still another study, when the G138R mutant was conditionally expressed in cells of the chondro-osteogenic lineage using the Dermo1 promoter, mutant mice were found to suffer from osteopenia (Watkins et al., 2011). Mechanistically, osteopenia was found to be due to high bone turnover, increased osteoclast-medicate bone resorption as a consequence of increased osteoclastogenesis, and defective osteoblast differentiation (Watkins et al., 2011). Thus, Cx43 mutants may cause impairments in multiple pathways involved in organogenesis. Collectively, mutant mouse models of ODDD have led to key discoveries that not only link Cx43 to diseases but address the need for a full complement of Cx43 function in several key developmental processes.

E. Next-Generation Human Models of Oculodentodigital Dysplasia

Further advancements in our understanding of the genetic and mechanistic basis of ODDD have now benefited from new models that may better represent this multiorgan-affected syndrome. To this end, ODDD patients worldwide have generously donated skin biopsies for the purpose of establishing patient-based dermal fibroblasts. Given that these patient-derived dermal fibroblast have one normal and one mutant

allele that each express Cx43 from its native promoter, it is expected that both gene transcription and translation would be as found in the patient. Importantly, it is also possible to obtain dermal fibroblasts from unaffected relatives of the patients in an attempt to control for genetic diversity found within the human population. By using this approach it was discovered that patients encoding the D3N and V216L Cx43 mutants have distinct changes in Cx43 phosphorylation and expression levels and these changes may lead to subclinical defects in wound repair (Churko et al., 2011). Other studies reported that dermal fibroblasts harboring D3N or V216L Cx43 mutants caused the reprogramming and expression of extracellular matrix genes that may serve key roles in skin wound repair (Esseltine et al., 2015). Further studies using dermal fibroblasts from patients harboring L7V, G138R, and G143S Cx43 mutants revealed that every mutation caused distinct molecular changes in Cx43 localization, assembly, or function (Kelly et al., 2016). In the case of G143S, it retained a functional state not unlike wild-type Cx43, raising questions as to the molecular mechanism responsible for this mutation to cause ODDD (Kelly et al., 2016). In summation, consistent with studies from reference cells and mutant mice, studies from patient-derived cells revealed that there appears to be several classes of Cx43 gene mutations that cause ODDD by distinct mechanisms leading to mutation-specific pathologies. Importantly, depending on how a specific mutation causes disease, they may be amenable for therapeutic interventions as we enter the dawn of new gene editing technologies.

Although ODDD remains a rare disease, understanding the etiology of this disease is critically important, because it involves mutations in the most commonly found connexin gene in humans, *GJA1*. Thus, understanding how mutations in this Cx43 encoding gene cause disease also provides critical insights into the role of Cx43 in any of the over 50 distinct cell types where it is expressed. Surprisingly, some of these Cx43-rich cells and tissues remain unaffected by loss- or gain-of-Cx43 function, further elucidating the redundancies and compensatory mechanisms that protect the human body from developmental abnormalities and disease. Looking forward, one wonders if we can effectively treat this genetic disease in patients where the disease appears to increase in severity during aging for reasons that remain poorly understood. Cx43-regulating therapeutic drugs remain sparse as they have begun to enter stage 2 and 3 clinical trials but have yet to become a mainstay in any disease or injury treatment (Kelly et al., 2016). Perhaps the future rests in gene editing, where CRISPR-Cas9 could be used to repair ODDD-linked *GJA1* gene mutations during development or in affected organs. To this end, we have now reprogrammed ODDD patient-derived fibroblasts into inducible pluripotent stem cells and are assessing the role of a full complement of functional Cx43 in osteogenesis, a process often affected in ODDD patients (Esseltine et al., 2017). Complementary approaches are also being developed to repair the *GJA1* gene in these patient-derived stem cells as a proof-of-principal that ODDD is a fixable syndrome. While it is still early days for therapeutic applications of gene repair, there is new hope that patients with defined *GJA1* mutations can be effectively treated in the decades to come.

VIII. Connexins in Cerebrovascular and Retinovascular Disease

The central nervous system (CNS) comprises multiple cell types arrayed in complex interconnections which require sustained and efficient vascular supply of oxygen, glucose and numerous nutrients and metabolites. One way these various cell types interact is through GJs and HCs in specific ways related to their functional roles (Decrock et al., 2015). Within the mammalian brain, the various cell types express over ten different connexins, providing a network of diverse and complex 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).

The vascular cells express connexins (Cx37, Cx40, Cx43) (De Bock et al., 2011, 2013b) that contribute to interendothelial coupling. These play an important role in blood-brain barrier function, as demonstrated using $Cx30^{-/-}$ mice crossed with $Cx43^{fl/fl}$/hGFAP-Cre (global Cx30 KO and astrocyte-specific Cx43 KO) (Ezan et al., 2012). These mice show compromise of the blood-brain barrier under stress due to increased hydrostatic vascular pressure and shear stress, swelling of astrocytic endfeet due to edema, as well as reduced expression of aquaporin-4, forming water channels, 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). Astrocytes are physically separated from vascular endothelial cells by a continuous basal lamina; hence, the connexins present at this endothelial-astrocyte interface do not form GJs, at least not under normal conditions. Although Cx43 expression is very low in blood-brain barrier endothelial cells under normal conditions, astrocytic endfeet display highly prominent Cx43 expression (Simard et al., 2003). Endfeet-located Cx43 forms GJs that couple adjacent endfeet that facilitate Ca^{2+} wave propagation along the astrocyte lining of small blood vessels (Mulligan and MacVicar, 2004). Although there is little evidence for endothelial-astrocytic GJs, pericytes also express Cx43,

which has been proposed to form GJs with astrocytes as well as endothelial cells (Wakui et al., 2006; Winkler et al., 2011). Specifically, pericytes contact endothelial cells via so called peg-and-socket junctions that are extensions from one cell invaginating another cell and that been reported to be immunopositive for connexins (Vigmond et al., 2000; Caruso et al., 2009; see also De Bock et al., 2014b).

The main parenchymal connexins, Cx43 and Cx30 (Nagy et al., 1999), are highly expressed in astrocytes, while Cx26 has also been reported (Nagy et al., 2001). GJs form a functional syncytium of coupled astrocytes, contributing to spatial buffering, in dealing with elevated concentrations of extracellular potassium ions (K^+) during increased neuronal activity; GJs assist in dispersal of K^+ accumulated by astrocytes (Walz and Hertz, 1983). The role of astrocytic Cx43 has been extensively examined in the context of GJs functioning to spatially buffer potassium and glutamate (Wallraff et al., 2006; Giaume et al., 2010), whereas Cx30 modulates astrocyte glutamate transport, thereby controlling hippocampal excitatory synaptic transmission (Pannasch et al., 2014). It was also shown that Cx30 controls the morphology of astrocytic processes at the synaptic cleft, and this was associated with altered glutamate clearance by astrocytes (Pannasch et al., 2014).

GJs in oligodendrocytes (Cx32, Cx29, Cx47) have been shown to be essential for proper myelination (Menichella et al., 2003), as well as potassium buffering (Menichella et al., 2006). Although microglia have been reported to express Cx43 and form GJs (Eugenín et al., 2001), others have not observed Cx43 immunoreactivity in microglia (Mei et al., 2010; Theodoric et al., 2012). Others have demonstrated that microglial Cx43 does not form GJs (Wasseff and Scherer, 2015) but rather HCs (Takeuchi et al., 2006). Expression of other connexins in microglia has also been described, including Cx32 (Dobrenis et al., 2005; Maezawa and Jin, 2010).

Depending upon the maturity of the CNS, neurons can express seven different connexins. These include Cx36, which is most highly expressed in neurons (Condorelli et al., 1998; Belluardo et al., 2000; Venance et al., 2000; Rash et al., 2012), as well as Cx30.2 (Kreuzberg et al., 2008), Cx31.1 (Dere et al., 2008), Cx40 (Personius et al., 2007), Cx45 (Li et al., 2008a), Cx50, and Cx57 in the retina (Puller et al., 2009). This presumably reflects the diversity of neuronal cell types, expressing a range of connexins with varying functions in the developing CNS. However, specific knockout of the prototypic neuronal Cx36 in mice results in near complete loss of neuronal GJC in the mature CNS, indicating that it is the primary neuronal connexin (Deans et al., 2001; Hormuzdi et al., 2001; Long et al., 2002; De Zeeuw et al., 2003).

A. Inflammation and Reduced Vascular Integrity in Cerebrovascular and Retinovascular Disease

When considering connexins in cerebrovascular and retinovascular disease, two pathogenic aspects appear to be important. The first aspect concerns inflammation and its effect on vascular integrity. The second is the switch from an initial inflammatory response to a self-perpetuating innate immune system, commonly involving the inflammasome pathway and resulting in a chronic inflammatory condition that is typical for chronic cerebrovascular and retinovascular disease. All of these have a connexin HC component, with interventions in a number of animal models improving outcomes, but with the role of direct cell-cell coupling via GJs being more complex. There may, for example, be multiple roles for connexins at the endothelial-glial interface, taking into account GJ coupling, HCs, and roles of connexins in pericytes and in endothelial mitochondria (Trudeau et al., 2012).

Two recent reviews detailed the conflicting evidence for the effect of inflammatory conditions on connexin transcription/protein translation and GJ function (Kim et al., 2016b; Willebrords et al., 2016). Analysis of publications cited in the Willebrords review shows that Cx43 levels (mRNA or protein) were *reduced* in 15 studies involving infection or the addition of inflammatory cytokines, but *increased* in 10 studies. Within a study, different cell types may respond differently. This variability in results reflects the diversity of models, cell types, time course, and dosing used and in vitro and in vivo responses may vary and even cells from different regions of a tissue may respond differently (Bennett et al., 2012). In addition, although "bystander death" is considered to be a GJ phenomenon [the term was originally coined to describe tumor cell-induced apoptosis in neighboring cells during coculture (Freeman et al., 1993)], Cx43 gene knockout and consequent loss of GJ coupling exacerbate injury size in the CNS (Siushansian et al., 2001; Nakase et al., 2003), indicating that maintenance of a functional astrocytic syncytium is essential or Cx43 has other non-channel-related functions.

On the other hand, connexin HCs, which are tightly controlled under normal conditions with low opening probability, appear to have a more consistent role in CNS inflammatory responses, forming a "pathologic pore" under injury or inflammatory conditions (Decrock et al., 2009b, 2015; Vinken, 2015). HC opening may provoke 1) ion fluxes that challenge osmoregulation; 2) the release of ATP, glutamate, and lactate that may contribute to neuronal loss; 3) breach of the blood-brain barrier or blood-retina barrier; and 4) initiation and perpetuation of inflammatory cycles. Once released, ATP triggers Ca^{2+} waves and further ATP release (Bennett et al., 2003; Neary et al., 2003; Suadicani et al., 2004; Scemes and Giaume, 2006), mediating

secondary damage distal to the trauma zone (Rovegno et al., 2015). In terms of inflammation, HC opening can be initiated by bacterial LPS or peptidoglycan (De Vuyst et al., 2007; Robertson et al., 2010), with HC opening considered to be an initiating step in the innate immune system. Release of ATP increases Toll-like receptor 2 and Il-6 production (Robertson et al., 2010) and UDP release may act as a danger signal from stressed or apoptotic cells (Qin et al., 2016). Inflammatory conditions typically promote HC opening while reducing GJ communication (see *section II.E.5.b*). Decreased cell-cell coupling may serve to deliberately isolate viral or bacterial infected cells. Increased vascular wall permeability correlates with a rapid immune response [such as neutrophil invasion (Cronin et al., 2008)], but vessel die back also isolates vessels from a possible source of infection, reducing the risk of septicemia. Inflammation is a double-edged sword though, and a hyperactive response exacerbates damage to both inflamed and surrounding tissue (Lehnardt et al., 2003). In the majority of wounds with modern hygiene, in most spinal cord and brain trauma (where the tissue is not exposed), and in virtually all CNS chronic disease conditions, this immune response becomes excessive or worse, perpetuates the inflammatory response, despite the absence of infection. Although pretreatment with the GJ blocker exacerbated infection in a model of peritonitis (Qin et al., 2016), no animal studies involving HC block have to date shown increased signs of infection.

B. Chronic Inflammatory Disease

Independently of GJ and HC roles in acute CNS injury, it is becoming apparent that perpetuation of chronic inflammatory conditions may be connexin HC mediated. Many chronic inflammatory diseases are associated with vascular dropout or degeneration. Vascular degeneration is a feature of inflammatory neurodegenerative diseases such as Alzheimer's and Parkinson's, with Alzheimer's the most common neurodegenerative condition seen in aging populations and Parkinson's second. In the eye, age-related macular degeneration affects as many as one in seven over the age of 50 in developed nations and diabetic retinopathy affects over 15% of Caucasians. Its prevalence is, however, three times higher in some populations such as New Zealand Māori and Pacific peoples. Macular edema and open angle glaucoma are likewise serious eye conditions associated with vascular degeneration. Although neovascularization is typical of the wet form of macular degeneration (about 10%–15% of all age-related macular degeneration) and diabetic retinopathy, inflammation and vessel dropout is increasingly considered to be the underlying cause of the disease and subsequent vision loss in those patients.

Chronic inflammation in these conditions would appear to involve a switch to the innate immune system's inflammasome pathways. ATP and its metabolic derivatives ADP, AMP, and adenosine are key inflammasome activators through P2XR, P1YR, and P1R receptor binding to trigger the NLRP3 inflammasome pathway (Riteau et al., 2012; Baron et al., 2015). Damage-associated molecular patterns (such as proinflammatory cytokines, chemokines, reactive oxygen species, and ATP), pathogen-associated molecular patterns (such as lipopolysaccharides, pathogens, irritants, and peptidiglycans), or TNF-α may activate the inflammasome pathway via the nuclear factor-κB complex. The end result is activation of caspase-1 and cleavage of the proinflammatory cytokines IL-1β and IL-18 into their mature forms and release from the cell. Increased TNF-α and IL-6 release also occurs, along with a new round of ATP release (for review, see Kim et al., 2016b; Willebrords et al., 2016; Zhou et al., 2016). There is a wealth of evidence for connexin HC roles in each of the inflammasome pathway stages: initiation, inflammatory cytokine release, and perpetuation. Connexin HC opening can facilitate the release of several molecules, including ATP, glutamate, aspartate, and lactate; the bidirectional flow of ions; and can lead to the onset of astroglial intercellular Ca^{2+} waves that contribute to neurodegeneration (Wang et al., 2013a; Castellano and Eugenin, 2014; Lohman and Isakson, 2014; De Bock et al., 2014a; Karagiannis et al., 2016). Furthermore, connexin HC opening probability is increased in response to injury and inflammation, and this has been correlated with the release of inflammatory cytokines such as TNF-α, IL-18, and IL-1β and the induction of IL-6 and TLR2 mRNA expression. If the innate immune response is unsuccessful in clearing a disease stimulus, short-term elevation of proinflammatory mediators can progress to chronic, self-perpetuating inflammation characteristic of the diseases mentioned above. It can be difficult to distinguish between GJ intercellular communication and HC roles in inflammatory disease, but analysis of studies cited in one recent review (Willebrords et al., 2016) showed that with disease, injury, or inflammatory cytokine addition, GJ intercellular coupling was decreased in 18 studies, with only 3 showing a coupling increase. Conversely, where HC activity was measured, all 12 studies revealed increased activity.

Both connexin HCs and the related pannexin channels may contribute to pathologic ATP release with the contribution that each makes often difficult to dissect (Bennett et al., 2012; Montero and Orellana, 2015). In one study though, pannexin channels were shown to be associated with ATP release during apoptosis but not for inflammasome activation (Qu et al., 2011). In an in vitro endothelial cell hypoxia model, two-thirds of the ATP release was through connexin HCs and one-third through pannexin channels, a combination of HC block [using Peptide 5 (O'Carroll et al., 2008)] and probenecid blocking all hypoxia-induced ATP release (and matching the effect of the nonspecific channel

blocker carbenoxolone) (Kim and Green, 2016). With reperfusion (return to normal medium), ATP continued to be released through connexin HCs but there was now no contribution from pannexin channels. In an Alzheimer's disease animal model, inflammation triggered pannexin channel activity but only within a subpopulation of reactive astrocytes in direct contact with amyloid plaques. Cx43 HCs were a wider contributor to ATP (and glutamate) release (Yi et al., 2016). In a series of studies (Cea et al., 2013, 2016a,c), inflammasome activation was demonstrated to be Cx43 and Cx45 HC mediated, leading to skeletal muscle atrophy in a Duchene muscular dystrophy model or after sustained high dose glucocorticoid treatments.

The immune system has a variety of defenses against pathogen-associated molecular patterns and damage-associated molecular patterns, and connexin expression and function has been documented in the immune and lymphatic system (Oviedo-Orta and Howard Evans, 2004; Neijssen et al., 2007). Cx43 appears to play a role in neutrophil recruitment and T cell-activation (Glass et al., 2015) and is involved in B cell activation, spreading, and migration (Machtaler et al., 2011). Knockdown of Cx43 in animal models of skin wound and spinal cord injury, for example, significantly reduces neutrophil counts and subsequent macrophage invasion into wound sites (Qiu et al., 2003; Cronin et al., 2008). Once the inflammasome is activated, maturation and release of proinflammatory cytokines (although too large to pass through the HC itself) are modulated by connexin HCs, and conversely, inflammatory cytokines increase HC activity. In a spinal cord contusion model, Peptide5 delivered by osmotic mini-pump to the injury site significantly reduced both TNF-α and IL-1β levels compared with untreated controls when assessed 8 hours after the injury (O'Carroll et al., 2013). There was less lesion spread in treated animal lesions, but IL-1β was at sham control levels. It is difficult to show if this is a direct HC modulatory effect or whether the lowered cytokine levels may result from reduced ATP release in the first place, but activated microglia are known to secrete TNF-α and IL-1β, which triggers HC opening in astrocytes (Bennett et al., 2012). The astrocytes then release ATP, causing further inflammasome activation, and increase extracellular glutamate by decreased uptake via Na^+-dependent transporters or directly release glutamate, which can kill neurons by excitotoxic signaling. Astrocytes are therefore both instigator and effector and key players in infectious and neurodegenerative diseases of the CNS (Kielian, 2008; Quintanilla et al., 2012). Figure 9 gives an overview of HC roles in injury spread and the perpetuation of chronic disease.

C. Central Nervous System Injury Models

1. Brain and Spinal Cord. Preterm ischemia is a cause of cerebral palsy. Studies in a large animal (sheep) model indicate a strong HC role in subsequent events. Although there is no evidence for a loss of vascular integrity, the model, with 24 hours-a-day, 7-day continuous recording of multiple parameters has provided insights into HC roles and treatment regimens for acute CNS injury. The model involves exteriorization of the lamb and the insertion of catheters or electrodes to enable measurement of mean arterial blood pressure and blood flow rate, heart rate, electroencephalographic activity, brain swelling, and body temperature. Inflatable carotid occluder cuffs are placed around the carotid arteries and a catheter is placed into the left lateral ventricle of the brain to allow treatment delivery (for further details, see Davidson et al., 2012b). In a variation of the model, preterm asphyxia was modeled in a similar manner but with an inflatable cuff placed around the umbilical cord instead of the carotid arteries. The carotid artery cuffs were inflated for 30 minutes to create severe ischemia, and then in the asphyxia model the umbilical cord cuff was inflated for 25 minutes.

Peptide5 delivery at high doses (1250 μmol/kg over 25 hours) was reported to have adverse effects, most likely attributable to GJ uncoupling. In those animals, greater brain swelling was evident, with a trend toward higher lactate concentrations and mortality (Davidson et al., 2012a). However, HC-blocking doses (50 μmol/kg for the first hour and 50 μmol/kg spread over the subsequent 24 hours) significantly improved outcomes (Davidson et al., 2012b). Electroencephalographic activity improved, seizures were reduced with less status epilepticus, there was earlier return to normal sleep cycling, increased survival of oligodendrocytes in intragyral and periventricular brain matter, and increased brain weight. All of those parameters were statistically significant with an intermediate increase in surviving neurons. After asphyxia in preterm fetuses, HC block with the same regimen gave earlier recovery of electroencephalographic power, was neuroprotective, and improved recovery of oligodendrocyte maturation (Davidson et al., 2014). With preterm ischemia, seizures and brain swelling starts about 6 hours, correlating with increased Cx43 expression, and in both models the peptide delivery was started 90 minutes after the insult to provide a therapeutic window of opportunity. When the start of treatment is delayed until 3 hours after the insult, HC blockade reduced seizure burden but no longer had effect on electroencephalographic power or histology (Davidson et al., 2015). These studies suggest that for these parameters at least, HC block but not GJ uncoupling may be crucial, and there is a window of opportunity for treatment that may be as short as 3 hours for acute injury [although this does not apply in chronic disease indications where connexin channel modulation treatment, in one report 8 weeks after injury (Ormonde et al., 2012), can be used to break the inflammatory cycle].

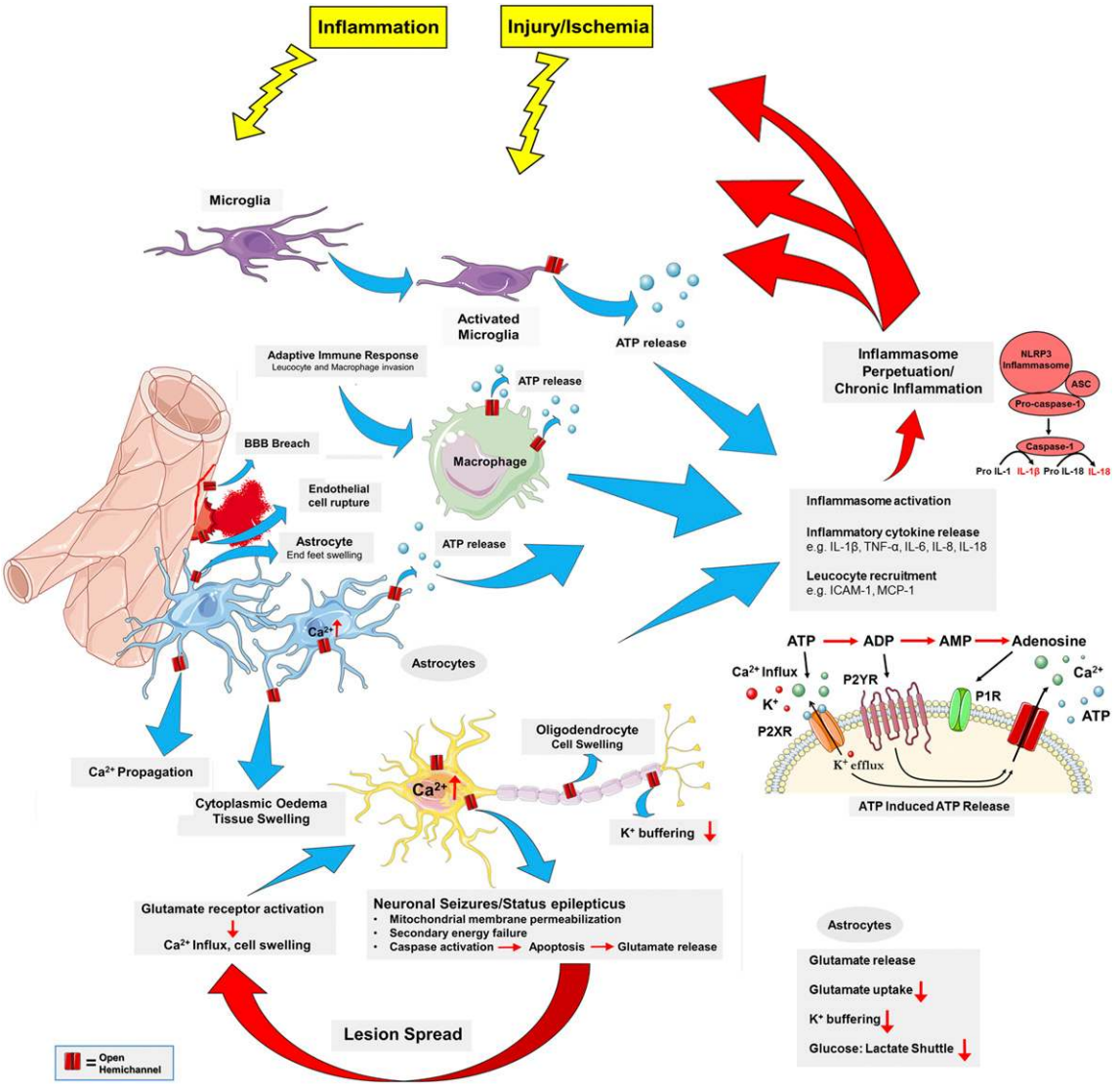

Fig. 9. Hemichannel-linked "pathologic pore" roles in injury spread and the perpetuation of chronic disease. Hemichannel opening plays a key role in cellular edema, lesion spread, initiation, and perpetuation of both the adaptive and innate immune responses and in particular in chronic disease conditions and the initiation and perpetuation of the inflammasome pathway. Multiple pathologic signals have been shown to trigger HC opening, resulting in pathologic levels of extracellular inflammatory stimulators such as ATP (with subsequent ATP-induced ATP release) and glutamate, the onset of calcium waves and seizures, and changes in the cytoplasmic ionic composition that may trigger apoptosis or loss of a cell's ability to osmoregulate. Loss of vascular integrity to breach the blood-brain or blood-retina barrier may be an initiating event, but conversely, inflammation leads to loss of vascular integrity, including rupture of endothelial cells and vascular dropout. The blue arrows to adjacent boxes indicate reported outcomes of HC opening in different cell types (irrespective of the specific connexin isoform expressed). Not all effects necessarily occur at once, depending upon the type and extent of injury and the specific cell type involved. These pathways have been implicated in a remarkable number of central nervous system cerebrovascular and retinovascular indications, including trauma, such as spinal cord injury; ischemia; stroke; and infectious disease and chronic diseases, including Alzheimer's, Parkinson's, amyotrophic lateral sclerosis, diabetic retinopathy, macular edema, age-related macular degeneration, and chronic pain. In several models, intervention using connexin HC blockers has been exploited to break the cycle, reducing the extent of damage after an acute insult or breaking the inflammatory cycle in chronic disease conditions. Red arrows at the (top) and (bottom) emphasize the HC-mediated feedback loops that contribute to damage spread and perpetuation of the inflammatory response.

Spinal cord injury intervention studies based on Cx43 knockdown with oligonucleotides have also revealed roles of connexins, as a protein or a channel, in CNS lesion spread, edema and inflammation, and in loss of vascular integrity that further exacerbate the damage. Spinal cord segments placed into organotypic culture swell despite the absence of a patent blood supply, and tissue dies back from the cut edges. Treated with Cx43-specific antisense oligodeoxynucleotides to block Cx43 protein translation, a reduction in segment swelling and improvement in neuron survival was observed, with ex vivo cord survival for up to 5 days (Zhang et al., 2010). This is also apparent with connexin mimetic peptide, with the ex vivo model in conjunction with in vitro cell culture models (dye uptake and dye spread) demonstrating that these effects are HC mediated (O'Carroll et al., 2008). In vivo, there are three rat spinal cord injury studies demonstrating that connexin channel modulation significantly improves outcomes. In the first, using Cx43-specific antisense

oligodeoxynucleotides after a compression injury, behavioral tests showed improved locomotion within 24 hours, with continued improvement for 4 weeks (when the study was terminated). Treated cords had significantly less edema and lesion spread, less inflammation (both neutrophil invasion and astrocytosis), and a dramatic reduction in vascular hemorrhage, which otherwise occurs for more than 4 mm either side of the injury site (Cronin et al., 2008). After partial spinal cord transection too, the Cx43 antisense treatment reduced inflammation, including microglial activation in and around the lesion site (Cronin et al., 2008). In a separate study, Cx43 mimetic peptides (Peptide5) also improved locomotor outcomes up to 5 weeks postcontusion injury. The treatments (delivered via an intrathecal catheter and osmotic minipump starting 1 hour postinjury) decreased levels of TNF-α and IL-1β in the wound bed 8 hours postinjury, with a subsequent reduction in astrocytosis and activated microglia as well as an increase in motoneuron survival (O'Carroll et al., 2013). Clinically this method of peptide delivery is less likely to be acceptable, and a third study, with a contusion injury, instead used systemic delivery (three intraperitoneal injections at 2-hour spacings) of the Cx43 mimetic Peptide5 (Mao et al., 2016). Treated rats showed improved locomotor activity and reduced at the level mechanical allodynia (central pain sensitization). Again lesion size was significantly reduced, astrocytic and activated macrophage and microglial responses were significantly reduced, and there was a significant sparing of neuronal cell numbers.

With respect to the inflammasome pathway specifically, as discussed above, a recent study provided evidence that spinal cord injury stimulates inflammasome activation at the injured site (Zendedel et al., 2016). The HC blocking peptides decrease levels of TNF-α and IL-1β, which are markers of inflammasome activation, in the wound bed 8 hours post-spinal cord injury (O'Carroll et al., 2013). This may result from a reduction in HC-mediated ATP release that triggers the inflammasome pathway. ATP release was also reduced after a weight drop spinal cord injury in a mouse model with astrocyte-specific deletion of Cx43 in astrocytes (Huang et al., 2012a). The blocked ATP release paralleled reduced inflammation (less astrogliosis and microglia activation), resulted in smaller lesions, and significantly improved motor recovery. Excessive ATP release from damaged tissue leads to activation of P2X7 receptors and systemic delivery of a P2X7 receptor antagonist (Brilliant Blue G) is similarly able to significantly improve outcomes, by acting downstream of the HC opening event (Wang et al., 2004; Peng et al., 2009). ATP released as result of Cx43 HC opening after spinal cord injury is associated with chronic pain as noted above in the Mao et al. (2016) contusion study. In animals with spinal nerve ligations, Cx43 siRNA downregulated Cx43 expression to alleviate mechanical hypersensitivity (Xu et al., 2014) and in weight drop experiments Cx43/Cx30 deletions (but not Cx30 deletions alone) prevented heat hyperplasia and mechanical allodynia. The change was associated with reduced astrogliosis, suggesting an important role for astrocytes and specifically astrocyte HC opening in chronic pain (Chen et al., 2014; for further discussion of Cx43 HC and GJ roles chronic neuropathic pain following spinal cord injury, see also Chen et al., 2012a). In another, very comprehensive study, satellite glial cells in the cervical C6-C8 region specifically exhibited increased GFAP and Cx43 expression associated with pain after a thoracic T3 compression or transection lesion (Lee-Kubli et al., 2016). Carbenoxolone or glycyrrhizic acid blocked the pain response, as did the connexin mimetic peptides Gap27 and Gap26, but not Gap19. These authors concluded that the pain response is GJ mediated, not HC mediated, since Gap19 (a HC specific peptide blocker, see *section III.B.2.a*) did not reduce paw withdrawal thresholds in pain testing. However, this in is contrast to other recent reports (Chen et al., 2014; Tonkin et al., 2016) where HCs have been implicated and may reflect difficulty in delivery of the cytoplasmic tail-acting Gap19 that needs to penetrate the plasma membrane to be effective; all of the other blockers used close HCs too.

2. Gap Junctions, Hemichannels, and Stroke. Stroke is the second leading cause of death worldwide and also contributes to a large health and economic cost due to stroke-related disability, rehabilitation, and long-term care (Lo et al., 2003; Feigin et al., 2014). Stroke causes neurologic deficits arising from an acute focal injury of the CNS due to a vascular event, leading to cerebral infarction, intracerebral hemorrhage, and subarachnoid hemorrhage (Sacco et al., 2013). Ischemic stroke results after a transient or permanent occlusion of an artery supplying the brain (Sacco et al., 2013). Since ischemic stroke accounts for approximately 80% of all strokes (Thrift et al., 2001), we will focus on this condition.

Stroke affects neurons, glia, vascular cells, and extracellular matrix components, which together influence mechanisms of tissue injury and repair taking place over an acute and progressive course. Within the ischemic tissue core, low ATP levels, ionic imbalance, and metabolic failure lead to rapid cell death in minutes (Lo et al., 2003). However, outside this core there is a peri-infarct region arising due to compromised blood supply, leading to milder ischemic conditions because of some compensation by collateral perfusion (Lo et al., 2003). Within this peri-infarct region, mechanisms associated with death and repair provide strategic therapeutic targets. Much work has focused directly on prevention of, or subsequent rescue of, neurons. Astrocytes within this region contribute to neuronal survival by maintaining the extracellular ionic environment, secreting growth factors and cytokines, and

stimulating angiogenesis (Chen and Swanson, 2003). Therefore, astrocytes provide an avenue to influence neuronal survival under ischemic injury.

During a stroke, minutes after ischemia, cellular energy is depleted and neurons and astrocytes in the ischemic core become unable to maintain transmembrane ionic gradients and die, resulting in damaged tissue, i.e., ischemia-induced cell death (Siesjö, 1992; Ginsberg, 1995). This damaged region will gradually expand to include cells in the surrounding penumbral area, which ultimately become irreversibly damaged (Hossmann, 1994). Clinical outcome of stroke and long-term prognosis are directly related to infarct size; thus larger infarcts have the worse outcome. Mechanisms that contribute to cell injury and death after ischemia are activated at the same time as endogenous neuroprotective pathways instrumental to cell survival and tissue repair. One potential contributor to ischemia-induced cell death is glutamate neurotoxicity (Chakravarthy et al., 1998; Vespa et al., 1998). Evidence indicates that ischemia-induced neurotoxicity can be the result of release of high amounts of glutamate (Rothman and Olney, 1986; Pellegrini-Giampietro et al., 1997). Cellular death associated with glutamate excitotoxicity occurs by two possible mechanisms: acute cellular swelling and late degeneration. In the first mechanism, glutamate activates ionotrophic receptors, which depolarizes the cell, resulting in cell swelling and, potentially, necrosis. Late neuronal degeneration is due to overstimulation of glutamate metabotropic receptors, resulting in massive Ca^{2+} influx and, ultimately, apoptosis.

Cx43 is the predominant GJ protein in astrocytes, and its expression is increased in reactive astrocytes in ischemic tissue (Hossain et al., 1994; Nakase et al., 2006, 2009). Cx43 membrane channels, both as GJs and HCs, impact numerous astrocytic processes involved in brain homeostasis and tissue repair (reviewed in (Freitas-Andrade and Naus, 2016). While Cx43 expression has been shown to be directly correlated with reactive astrogliosis in brain injury (Theodoric et al., 2012); the respective contributions of GJs versus HCs were not examined. Evidence from several sources supports the importance of the astrocytic environment, particularly astrocytic networks formed by GJs, in playing a critical role in neuroprotection and neurodegeneration (Giaume et al., 2010). In contrast, enhanced Cx43 expression can also result in increased HCs in astrocytes, which generally is associated with increased cell injury and death (Giaume et al., 2013).

Spatial buffering of the extracellular environment is mediated through GJs between astrocytes. Thus astroglial Cx43 has been shown to be associated with neuroprotection and astrocyte survival in experimental stroke conditions (Naus et al., 2001; Siushansian et al., 2001; Nakase et al., 2003, 2004; Kozoriz et al., 2010, 2013; Le et al., 2014; Shinotsuka et al., 2014). Although many studies have focused on the use of in vitro models to mimic stroke conditions, in vivo studies more closely represent the clinical situation. In this regard, transgenic and knockout mice have been extremely useful to explore the role of GJs and HCs in stroke. To specifically address the role of Cx43, studies were carried out on Cx43 knockout mice. Unfortunately, these homozygous Cx43 null mice ($Cx43^{-/-}$) die shortly after birth due to a cardiac malformation (Reaume et al., 1995). In contrast, heterozygous null mice ($Cx43^{+/-}$) survive and appear healthy, despite 50% reduction in Cx43 expression. When these heterozygous null mice were subjected to permanent middle cerebral artery occlusion (pMCAO), they exhibited a significant increase in infarct volume compared with wild-type controls (Siushansian et al., 2001), suggesting that disruption of the astrocytic gap junctional syncytium mediated by Cx43 is detrimental to neuronal survival in ischemic stroke conditions. However, since Cx43 is ubiquitously expressed, the effects observed in the heterozygous null mice may be due to other contributing systemic factors that are affected by the deletion. To address this issue, Nakase et al. (2004) investigated specifically the role of astroglial Cx43 in stroke using a conditional Cx43 knockout mouse (GFAP-Cre;$Cx43^{fl/fl}$). These Cx43 conditional knockout mice, lacking Cx43 in astrocytes, also exhibited a significant increase in stroke volume and enhanced apoptosis compared with wild-type controls when subjected to pMCAO (Nakase et al., 2004). Interestingly, a higher level of inflammation, as measured by reactive microglial cells, was also found in the Cx43 conditional knockout mice 4 days after pMCAO (Nakase et al., 2004). This finding highlights a possible link between astrocytic Cx43 expression and inflammation in ischemic conditions (Nakase et al., 2004). Several groups have shown that exposing astrocytes to inflammatory factors can induce Cx43 HC activity and affect neuronal viability in neuron/astrocyte coculture conditions (Retamal et al., 2007a; Froger et al., 2010; Orellana et al., 2011a; Boulay et al., 2016).

Various in vitro stroke models are well suited to address mechanisms regarding the roles of GJs and HCs. For example, hypoxia-reoxygenation along with elevated glucose concentration activates Cx43 HCs, resulting in astrocyte cell death (Orellana et al., 2010). In contrast, under the same conditions astrocyte cell death was reduced by the HC blockers, Gap26, Gap27, or La^{3+}, applied at the start of the reoxygenation period. Moreover, the p38 MAPK inhibitor, 4-(4-Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole (SB202190), which reduces hypoxia-reoxygenation opening of Cx43 HCs, was also protective (Orellana et al., 2010). These results suggest ischemic conditions involving both inflammation and hypoxia synergistically promote Cx43 HC activity, contributing to both astrocyte and neuronal cell death.

The deleterious effects of Cx43 HCs in stroke were further investigated in vivo using a mouse ODDD model (Kozoriz et al., 2013) (see *section VII*). The common external features in this syndrome, which is caused by a number of Cx43 mutations (Laird, 2014), include ocular, nasal, dental, and digital abnormalities, and can include neurologic deficits (Paznekas et al., 2009). Many of these Cx43 mutations are associated with reduced GJ formation and increased HC activity (McLachlan et al., 2005; Dobrowolski et al., 2007; Kozoriz et al., 2013). One mouse model of this disorder was created with a missense G60S point mutation (Flenniken et al., 2005); the phenotype of $Cx43^{+/G60S}$ mice is similar to human ODDD. Since the G60S mutation reduces the level of Cx43 and GJ coupling in astrocytes and also leads to increased HC activity, it is not surprising that these mice show a significant increase in infarct volume pMCAO (Kozoriz et al., 2013). A similar stroke study in mice expressing a C-terminal truncated Cx43, K258stop (Maass et al., 2004), also demonstrated an increase in stroke injury that was likewise associated with enhanced HC activation (Kozoriz et al., 2010). Taken together, these studies support the role of Cx43 HCs as a key focal point in stroke.

Although many studies have highlighted the role of GJs in neuroprotection, there are also several reports demonstrating that GJs also play a role in enhancing cellular injury (Cotrina et al., 1998; Lin et al., 1998; Rami et al., 2001; Nodin et al., 2005; Perez Velazquez et al., 2006). Under ischemic in vitro conditions astrocytes show reduction in coupling; however, they do not uncouple during or after a lethal injury (Cotrina et al., 1998). This raises the possibility that excess secondary intracellular messengers, such as Ca^{2+} and IP_3, can migrate freely between dying astrocytes into healthy cells and extend the focal injury (Cotrina et al., 1998). Furthermore, administration of octanol (GJ inhibitor) led to significant reduction of Cx43 immunoreactivity and reduced neuronal death in the CA1-CA2 regions of ischemic animals (Rami et al., 2001). These studies suggest that reduced GJ communication may inhibit the flow of undesirable metabolites that could potentially cause neuronal damage in the peri-infarct area (Rami et al., 2001). In addition to the possible propagation of intercellular cytotoxic substances, spreading depression-like depolarizations through open GJs may also contribute to neuronal loss (Cotrina et al., 1998; Lin et al., 1998; Perez Velazquez et al., 2006). It is not understood when propagation of harmful or protective molecules takes precedence. Differences in experimental design, brain region-dependent vulnerability to ischemia, and the type of connexins and pannexins under study may explain why these GJ proteins have been observed to have either protective or destructive roles in the CNS.

Others have shown, however, that there are also reactive astrocytes with decreased Cx43 expression surrounding the infarct (Hossain et al., 1994; Cotrina et al., 1998). Furthermore, Cx43 expression in reactive astrocytes is spatially heterogeneous around cortical lesions induced by kainic acid excitotoxicity (Sawchuk et al., 1995; Koulakoff et al., 2008; Gangoso et al., 2012). This heterogeneity in Cx43 expression, in reactive astrocytes, may reflect the different astrocytic populations, with distinct molecular signatures, residing within specific brain regions (Wallraff et al., 2004). In addition, damaged ischemic neurons with varying degrees of injury may participate in regulating the surrounding astrocytic GJs. Indeed, others have shown that paracrine-secreted factors can regulate astrocyte GJs (Blomstrand et al., 2004; Koulakoff et al., 2008; Wuestefeld et al., 2012). The interplay between paracrine activation and astrocyte-type specific responses with respect to Cx43 expression/activity is not fully elucidated.

Various potential therapeutic interventions are currently being examined to reduce neuronal death associated with stroke. Such neuroprotective therapies include glutamate release inhibitors and receptor antagonists, Ca^{2+} channel blockers, GABA receptor agonists, gangliosides, neurotrophic factors, calpain inhibitors, caspase inhibitors, free radical scavengers, and immuno- and cell metabolism modulators. Very few therapeutic avenues have proven successful to treat stroke [for recent review, see Cook and Tymianski, 2011). One noteworthy success has used of a small peptide that prevents postsynaptic density protein PSD-95 from activating NMDA receptor-mediated neurotoxicity pathways (Cook et al., 2012; Hill, 2012). This establishes the validity of therapies specifically targeting interacting domains of key signaling proteins and holds promise for clinical applications, particularly related to stroke. Small biomolecules (antisense RNA, peptides) targeting GJs are in fact currently in clinical trials for healing chronic wounds and corneal injuries, providing proof of principle for these channels as therapeutic targets (Butera et al., 2009; Ormonde et al., 2012). In this context, Cx43 provides a substrate for therapeutic strategies targeted to astrocytes rather than neurons, based on the premise that both cell types play critical roles in stroke recovery. One such therapeutic is the peptide Gap19, which has been shown to block HCs but not GJ channels in astrocytes (Wang et al., 2013c; Abudara et al., 2014). It is envisioned that a therapeutic approach targeting both neuronal signaling pathways and astrocyte-mediated events should enhance protection after stroke.

3. Ocular Models and Disease. The link between injury and degenerative disease and loss of vascular integrity is more easily visualized in the eye. Ex vivo optic nerve and in vivo retinal injury models all demonstrate the link between inflammation and vessel leak. Isolated rat optic segments exposed to oxygen glucose deprivation for 2 hours and then placed into a

normoxic air-liquid interface organotypic culture will swell and show an inflammatory response (increased GFAP expression and microglia activation). In addition, blood vessels become fragmented (Danesh-Meyer et al., 2008). Treatment with Cx43 antisense reduces edema and inflammation (both GFAP levels and number of activated microglia), and despite the absence of blood flow in the organotypic culture model, blood vessels retain greater structural integrity. In vivo, retinal ischemia is known to cause vascular leakage (Kaur et al., 2008; Abcouwer et al., 2010), with loss of vascular integrity beginning in the first few hours after ischemia but lasting for up to 2 months (Wilson et al., 1995). To establish the mechanism of vessel leak, a high intraocular pressure model of ischemia-reperfusion was used (Danesh-Meyer et al., 2012). A cannula in the anterior chamber of the eye was used to raise intraocular pressure to 120 mmHg for 1 hour and then removed to allow reperfusion. Although the pressure was applied uniformly across the whole retina, and the whole retina is made ischemic, inflammation and Cx43 levels increased, starting within 2 hours, but only in small patches and adjacent to blood vessels. Evans Blue dye perfused through the animal demonstrated vascular leak in a similar pattern, starting 1–2 hours after reperfusion and indicating that vessel leak may precede inflammation, which is the result, in this case, of blood-retina barrier breaches. Confocal microscopy revealed astrocytic processes crossing the lumen of vessels, indicating that endothelial cells had burst, the authors proposing this being due to HC opening and an inability to osmoregulate leading to rupture. Labeling of vessels 4 hours after injury indicated the vascular bed was badly disrupted with blind endings and collapsed vessels apparent. In three separate studies, delivery of the peptidomimetic Peptide5 reduced vascular leak (up to 86% at the peak 4 hours post-reperfusion time point), reduced inflammation, and downstream (followed for up to 4 weeks later) resulted in highly significant neuronal sparing (greater than 50% sparing in all three studies), which also has relevance for glaucoma (Chen et al., 2015b). The peptide was delivered systemically (Danesh-Meyer et al., 2012) by intravitreal injection into the eye in native form or encapsulated into slow-release poly(lactic-coglycolic) acid nanoparticles (Chen et al., 2015d) or lipoamino acid-modified to increase stability (Chen et al., 2015c). In all three studies, a single peptide injection at the point of reperfusion was given and at a concentration expected to block HCs with minimal effect on GJ coupling. Connexin isoforms other than Cx43 can play a role in secondary cell death, which may offer other points of intervention. Under excitotoxic conditions in the retina (exposure to NMDA), genetic deletion of Cx36 increases neuronal survival by up to 50%, whereas Cx45 deletion shows no benefit. Conversely, Cx45 deletion can reduce neuronal loss after an ischemic insult, but ablation of Cx36 is ineffective in this condition (Akopian et al., 2014). These latter studies, however, do not enable differentiation between GJ- or HC-mediated events.

In the retina ischemia-reperfusion studies above (Danesh-Meyer et al., 2012), breach of the blood-retina barrier appeared to be the instigating event. In a different model, a bright light damage model, inflammation appears to be the initiating factor. In this model albino rats are exposed to intense fluorescent light for 24 hours, resulting in retinal degeneration that mimics that seen in age-related macular degeneration, although at a faster rate. Photoreceptors and retinal pigment epithelium are lost, breakages appear in Bruch's membrane, and choroidal damage occurs with oxidative stress in choroidal endothelial cells (the eyes' main blood supply)(Wu et al., 2005; Marc et al., 2008). Cx43 increases after damage in parallel with inflammation, starting in the choroid first and them progressing into the retina (Guo et al., 2014). The levels of Cx43, remaining high over the first 2 days and only decreasing after 7 days, correlated with oxidative stress and macrophage invasion, suggesting a close relationship between Cx43 and the inflammatory process. In a follow up study (Guo et al., 2016), Cx43 HCs were blocked with two intravitreal injections of Cx43 mimetic Peptide5, one 2 hours into the 24-hour bright light period and one at the end of the bright light period. Such treatment resulted in reduced choroidal inflammation and improved functional recovery of both photoreceptors and neurons as judged from electroretinograms (mixed a-wave and mixed b-wave amplitudes, isolated rod PII and PIII amplitudes, and cone PII responses).

Another ocular disease associated with inflammation and vessel hemorrhage is diabetic retinopathy. While there are reports that Cx43 is downregulated in diabetes that may contribute to vascular degeneration (Tien et al., 2014, 2016), the methods used were indirect. In a recent study, Cx43 levels in wild-type, Akita, and Akimba mice were directly analyzed. The Akita mouse is diabetic but does not form diabetic retinopathy lesions at the ages studied. In that strain Cx43 levels appeared lower in the retina. In Akimba mice, however, which do develop signs of diabetic retinopathy, Cx43 was threefold higher in the ganglion cell layer, mainly associated with astrocytes, with increased GFAP expression in several retinal layers suggesting Müller cell activation. Importantly, there was increased blood vessel density, implying neovascularization with significantly higher Cx43 levels in vascular endothelial cells (Danesh-Meyer et al., 2016). Obviously, Cx43 labeling is not a marker for HC opening, and further interventional studies are needed. It is of note that the retinal ischemia-reperfusion model described above (Danesh-Meyer et al., 2012) has also been described as a model for diabetic retinopathy, because changes observed mimic those seen in the disease (Zheng et al., 2007; Wang et al., 2011). In human donor tissues, in which

diabetic retinopathy was confirmed with ex vivo fundus and optical coherence tomography imaging, Cx43 is highly upregulated compared with normal, age-matched donor tissues and especially high in the endothelium of blood vessels in regions of neovascularization, the vessels that hemorrhage as the disease progresses (Danesh-Meyer et al., 2016). All of these studies support the view that connexin HC regulation may have significant treatment potential for retinal diseases such as age-related macular degeneration, diabetic retinopathy, and macular edema. This may either be through the maintenance of vascular integrity or by damping down the inflammatory process, especially the inflammasome pathway, which is known to be a feature of these diseases (Tseng et al., 2013; Qi et al., 2014; Chi et al., 2015; Gao et al., 2015).

4. Neurodegenerative Brain Disease. Although neurodegenerative brain diseases are not strictly vascular diseases, several of them are associated with inflammation and loss of vascular integrity, and we therefore briefly discuss the role of connexins in these diseases. Alzheimer's disease, although noted for the accumulation of amyloid-β beta plaques and neurofibrillary tangles, is characterized by chronic brain inflammation and vascular dropout (Farkas and Luiten, 2001). In animal models, controlling leukocyte-endothelial interactions inhibits both amyloid deposition and reduces memory loss in those animals, suggesting that cerebrovascular disease is upstream of amyloid deposition and the formation of neurofibrillary tangles (Zenaro et al., 2016). Indeed vascular dysfunction diminishes brain capillary flow, reducing amyloid-β clearance and leading to neuronal dysfunction (Janota et al., 2016), and mutations known to provoke inflammatory reactions are proposed to cause microvasculature damage leading to dementia (Marchesi, 2016). Once formed though, amyloid-β may further exacerbate injury by enhancing connexin43 expression (Nagy et al., 1996). In Parkinson's disease, another chronic inflammatory disorder, there is small vessel dropout in brain regions affected by the disease (Guan et al., 2013), and in Lewy body dementia, reduced microvessel density, rather than vasoconstriction, is said to be the cause of reduced occipital blood flow (Miners et al., 2014). Furthermore, because the chronic inflammation cannot be contained, there is a link between diseases, with circulating inflammatory cytokines triggering disease elsewhere (Green and Nicholson, 2008). In a recent 2016 example (Yeh et al., 2016), the Parkinson's disease-free survival rate was significantly lower for patients who had spinal cord injury than for uninjured patients, with almost double the rate of Parkinson's onset in the spinal cord injury group.

The link between connexin channels and neurodegenerative disease is very strong, especially connexin HCs but also pannexin channels in inflammatory responses of glia and neurons (Orellana et al., 2011b, 2012; Bennett et al., 2012; Retamal, 2014). GJ inhibitors, and in particular connexin HC blockers, suppress excessive microglial glutamate release and have improved outcomes in animal models of multiple sclerosis, amyotrophic lateral sclerosis, and Alzheimer's disease (Takeuchi et al., 2011; Takeuchi and Suzumura, 2014; Yi et al., 2016). HC opening in astrocytes and microglia appears to initiate a "vicious cycle" of excessive ATP release underlying cerebral palsy resulting from preterm ischemia (Mallard et al., 2014), and the term HC-mediated "vicious cycle" emerges again in a murine model of Alzheimer's disease (Yi et al., 2016). In this very thorough study, the expression of connexins was shown to be upregulated in both human donor brain tissue and in an APP/PS1 mouse model of Alzheimer's disease. In acute hippocampal slices of tissue containing amyloid plaques, GJ communication was not altered (assessed using fluorescence recovery after photobleaching) but HCs were activated in astrocytes (assessed using EtBr dye uptake). A number of GJ (CBX, Gap26), HC (La^{3+}), or pannexin channel (^{10}Panx1, probenecid) blockers were used, as well as astroglial targeted Cx43 gene knockout. A minor pannexin channel component was observed in astrocytes immediately contacting the amyloid plaques, but connexin HC opening was widespread, leading to the release of ATP and glutamate. This maintained "a high calcium level in astrocytes placing them in the center of a vicious circle." With targeted knockout of Cx43 in astrocytes, gliotransmitter release was diminished, reducing oxidative stress and damage to hippocampal neurons associated with amyloid plaques. In two other transgenic models, mice carrying mutated human superoxide dismutase 1 as an amyotrophic lateral sclerosis model and double transgenic mice expressing mutated human amyloid precursor protein and presenilin 1 as an Alzheimer's disease model, a modified glycyrrhetinic acid connexin channel blocker inhibited excessive glutamate release from activated microglia (Takeuchi et al., 2011). Those authors too concluded that HC block may be effective in preventing microglia-mediated neuronal death in neurodegenerative diseases.

In vitro models have produced significant information on GJ and HC roles in cerebrovascular and retinovascular disease, although results can appear conflicting owing to the diversity of models, cell types used, time course, and dosing. Many of these may not always have relevance to the in vivo situation during an acute injury phase or in chronic disease. Nonetheless, patterns are emerging and increasing numbers of animal models, with improved channel blockers and knowledge of their pharmacodynamics available, are leading to consistency in demonstrating pathologic opening of connexin HCs and their role in inflammation, lesion spread, edema, loss of vascular integrity, and neuronal degeneration. More importantly, HC opening is

increasingly associated with perpetuation of inflammation, in particular the inflammasome pathway, in chronic disease. This has already been implicated in a remarkable number of CNS cerebrovascular and retinovascular indications including trauma, such as spinal cord injury or stroke, and chronic diseases including Alzheimer's, Parkinson's, amyotrophic lateral sclerosis, multiple sclerosis, diabetic retinopathy, macular edema, age-related macular degeneration, and chronic pain. While the field could still benefit from more specific reagents, a number of those existing are showing significantly improved outcomes in animal models where the therapeutic target is identified. Hopefully clinical advances can now be made for these indications where there is an unmet need for intervention.

IX. Concluding Remarks

In recent years we have seen the appearance of a number of interesting peptides such as L2, Gap19, Peptide5, AAP10 and its D-amino acid version rotigaptide (ZP123), and αCT1 composed of Cx43 CT9 peptide fused to an antennapedia membrane translocation motif. The mother peptide L2 and the pharmacophore-based peptidomimetic molecule ZP2519 target the Cx43 CT and prevent Cx43 GJ closure at low pH, as occurs in ischemia. Thus, ZP2519 has potential translational value for preventing postischemic GJ closure and arrhythmias. Interestingly, L2 and the L2 subdomain peptide Gap19 are blockers of Cx43-based HCs. L2 thus combines two interesting actions: it prevents acidification-induced GJ closure and inhibits HC opening. It would be interesting to test whether ZP2519 retains the HC-inhibiting properties of the mother peptide L2. AAP10 and ZP123 promote coupling via Cx43- and Cx45-based GJs and are interesting substances for the treatment of ischemia-linked arrhythmias. A phase II clinical trial investigating the safety and tolerability of ZP123 in patients suffering from unstable angina or myocardial infarction has been initiated (Kjølbye et al., 2007), but despite promising results, it was terminated (Zhang and Xiang, 2009). αCT1 peptide has substantial promise because it promotes GJs not by acting on the channels per se but by promoting the incorporation of Cx43 HCs in GJ plaques.

In terms of vascular disease, animal studies have revealed important and diverse contributions of the vascular connexins Cx37, Cx40, and Cx43 to atherogenesis, with Cx43 having an overall atherogenic effect while Cx40 protects against atherosclerosis and loss of Cx37 promotes atherosclerosis. Several human Cx37 SNPs have been identified, and those affecting connexin GJ or HC function appear to link to cardiovascular disease risk. Mice cardiac ischemia-reperfusion studies have unveiled a protective role of vascular endothelial Cx40, which limits neutrophil infiltration and inflammation in the reperfusion phase. In the heart, ischemic preconditioning and the role of mitochondrial Cx43 therein remains an interesting field to be further explored toward development of peptides and drugs that target this intracellular connexin pool. In addition to GJs and plasma membrane HCs, mitochondrial Cx43 should be considered as a third level for pharmacotherapeutic intervention. Cardiac fibrosis appears to involve ATP release through Cx43- and Cx45-based HCs in cardiac fibroblasts (Lu et al., 2012a) and fibroblast-cardiomyocyte GJ coupling may influence electrical signaling (Ongstad and Kohl, 2016), putting fibroblast connexins as another potential target in view. In terms of cardiac arrhythmias, alterations in the distribution and the abundance of GJs composed of Cx40 and Cx43 resulting from heart failure or other cardiac pathologies may lead to arrhythmogenesis. It remains to be seen whether HCs may contribute as an additional arrhythmogenic channel as recently suggested based on work with Gap19 and other peptides (González et al., 2015).

In cerebrovascular and retinovascular disease, HC-blocking peptides such as Peptide5 come up as interesting tools to reduce inflammation and preserve vascular integrity. Connexins in the vascular endothelium are intrinsically an utmost interesting target, because blood cells and endothelial cells are the first line to welcome a drug after absorption in the gastrointestinal tract. Obviously, we need more and better tools to target specific connexins and specific channels: GJs or HCs. These tools are urgently needed to understand better and distinguish HC functions from those of GJs. However, in terms of therapeutic application, the most specific tools not always make the best drugs. Spinal cord injury, stroke, diabetic retinopathy, macular edema, and age-related macular degeneration come up as diseases where novel connexin-targeting drugs would be very much welcome. There is currently a growing interest in connexin-targeting drugs for applications to skin, wound healing, and ocular disease (Ghatnekar et al., 2009; Grupcheva et al., 2012; Grek et al., 2015; Becker et al., 2016; reviewed in Danesh-Meyer et al., 2016; Naus and Giaume, 2016). Additionally, some existing drugs are repurposed to exploit their effects on connexins in a combinatorial approach, as, e.g., in the combination of modafinil with the GJ-inhibiting properties of flecainide to treat narcolepsy (Duchêne et al., 2016). Clearly, these examples just mark the start of a rapidly expanding interest in connexins as a potential pharmacotherapeutic target. At this stage, two important points on the agenda to get the field forward are 1) to better understand why EL interactions upon HC docking and formation of a GJ opens HCs, whereas applying peptides mimicking crucial EL domains closes unapposed HCs, and 2) why Cx43 loop-tail (CT-CL) interactions have a completely opposite effect on HCs compared with GJs. Increased

insights in these important questions will be helpful in designing novel molecules that target connexin channels from the outside or inside of the cell.

Conflict of Interest

Acknowledgments

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