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Review

Connexin-related signaling in cell death: to live or let die?

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Evidence is accumulating that some forms of cell death, like apoptosis, are not only governed by the complex interplay between extracellular and intracellular signals but are also strongly influenced by intercellular communicative networks. The latter is provided by arrays of channels consisting of connexin proteins, with gap junctions directly connecting the cytoplasm of neighboring cells and hemichannels positioned as pores that link the cytoplasm to the extracellular environment. The role of gap junctions in cell death communication has received considerable interest and recently hemichannels have joined in as potentially toxic pores adding their part to the cell death process. However, despite a large body of existing evidence, especially for gap junctions, the exact contribution of the connexin channel family still remains controversial, as both gap junctions and hemichannels may furnish cell death as well as cell survival signals. An additional layer of complexity is formed by the fact that connexin proteins as such, beyond their channel function, may influence the cell death process. We here review the current knowledge on connexins and their channels in cell death and specifically address the molecular mechanisms that underlie connexin-related signaling. We also briefly focus on pannexins, a novel set of connexin-like proteins that have been implicated in cellular responses to pathological insults.
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In multicellular organisms, the maintenance of tissue homeostasis ultimately relies on the critical balance between cell growth and cell death. Among the various cell death types, apoptosis has been most extensively characterized. Being the conceptual counterpart of necrosis, apoptosis is a genetically programmed and well-orchestrated process of selective cell deletion that occurs in all tissues as part of the normal cellular turnover.[1] It is also involved in a growing number of pathological conditions, such as in ischemia-related cell injury following stroke.[2,3] Two major apoptotic pathways can be distinguished, the mitochondria-mediated intrinsic cascade and the death receptor-mediated extrinsic pathway.[1,4–6] Both rely on the proteolytic activity of an evolutionary conserved family of cysteine proteases – the caspases – which form the biochemical basis of the apoptotic phenotype.[7] They are responsible for the cleavage of a large number of cellular proteins including major cytoplasmic and nuclear elements. The extrinsic signaling pathway is triggered by the binding of an extracellular death ligand, such as Fas ligand, tumor necrosis factor α (TNFα) or TNF-related apoptosis-inducing ligand (TRAIL) to their corresponding receptors at the plasma membrane (PM).[1,4–6] In contrast, the intrinsic signaling pathway is mediated by mitochondria and involves a diverse array of non-receptor-mediated stimuli that produce intracellular signals directly acting on targets within the cell. It is regulated by the B-cell lymphoma-2 (Bcl-2) family of pro- and anti-apoptotic proteins.[8] Necrotic cell death, on the other hand, has been often considered to be a passive process,

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Abbreviations: ATP, adenosine triphosphate; Bcl-2, B-cell lymphoma-2; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; Cx, connexin; CytC, cytochrome C; ER, endoplasmic reticulum; GCV, ganciclovir; GJ, gap junction; GJIC, gap junctional intercellular communication; HC, hemichannel; HSV-tk, *herpes simplex* virus-thymidine kinase; IP_3, inositol trisphosphate; MEK/ERK, mitogen-activated protein kinase kinase/extracellular signal-regulated kinase; NAD^+, nicotinamide adenine dinucleotide; NO, nitric oxide; OGD, oxygen/glucose deprivation; P_2X_7R, P_2X_7 receptor; $p90^{RSK}$, p90 ribosomal S6 kinase; Panx, pannexin; pC/EBPβ, phosphorylated CCAAT/enhancer-binding protein β; PM, plasma membrane; ROS, reactive oxygen species; TNFα, tumor necrosis factor α; TRAIL, TNF-related apoptosis-inducing ligand
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lacking underlying signaling events and occurring under extreme physico-chemical conditions, including abrupt anoxia, sudden shortage of nutrients, heat or detergents. Recent evidence, however, suggests that necrotic cell death can also be the morphological manifestation of a molecularly regulated event associated with pathologies such as ischemia-reperfusion injury, neurodegeneration and pathogen infection.[9,10]

In contrast to the wealth of knowledge concerning the extracellular and intracellular signaling cascades that govern (apoptotic) cell death, our understanding of the role of intercellular (cell-to-cell) communication in this process is still in its infancy. The most direct form of intercellular communication proceeds through gap junction (GJ) channels. These channels arise from the head-to-head interaction of two hemichannels (HCs) (connexons) on adjacent cells, which are hexameric channels composed of connexins (Cxs). The Cx protein has a four membrane-spanning topology with two extracellular loops, one cytoplasmic loop and a cytoplasmic N- and C-terminal region (Figure 1).[11–13] At present, more than twenty Cx species have been cloned from rodents and human and are named according to their molecular weight.[14] Gap junctional intercellular communication (GJIC) is driven by the passive diffusion of small ($<$1–1.5 kDa) hydrophilic molecules such as glucose, glutamate, glutathione, cyclic adenosine monophosphate (cAMP), adenosine triphosphate (ATP), inositol trisphosphate (IP_3) and ions (e.g., Ca^{2+}, K^+, Na^+).[13,15] The biophysical permeation properties of these substances depend on the nature of the Cx species that form the channel and clear differences in channel permeability have been shown for various ions, reporter dyes and signaling molecules such as cAMP or ATP.[16–19] The gating of GJ channels is controlled by a number of factors, with as prominent players the transmembrane voltage (over the PM), transjunctional voltage (over the GJ), Ca^{2+} and the phosphorylation status.[20–22] With the notable exception of Cx26, all Cxs are phosphoproteins that are targeted, particularly at their C-terminal tail, by a broad panel of kinases. HCs reside as closed channels in the PM but open by a process of 'loop gating' when their extracellular loops interact to form a functional GJ channel.[20,23] Before being incorporated into GJs, HCs can also be opened by various signals or conditions, including membrane depolarization,[24] a decrease of extracellular Ca^{2+},[25] cytoplasmic Ca^{2+} changes,[26] mechanical stimulation,[27] changes in phosphorylation status,[28] changes in redox status,[29] reactive oxygen species (ROS),[30] nitrosylation of the Cx protein,[31] ischemia/hypoxia,[3,32,33] and also certain Cx mutations.[34–37] Open HCs allow the entry of below 1 to 1.5 kDa substances (e.g., Ca^{2+}, Na^+)[38] or the escape of essential metabolites such as nicotinamide adenine dinucleotide (NAD^+),[39] ATP,[40] glutamate,[41] prostaglandins[42] and glutathione.[43]

Another family of GJ channel-forming proteins, the 'innexins', was first reported in invertebrates and later renamed 'pannexins' (Panxs) after their orthologs were discovered in vertebrates.[44] Thus far, three Panxs (Panx1, Panx2 and Panx3) have been characterized in rodents and human. Despite the lack of sequence homology between Cxs and Panxs, they share a similar topology and also display cell-specific expression patterns.[45] They appear to be endowed with several other Cx-like properties such as the ability to form homomeric Panx1 or heteromeric Panx1/Panx2 HCs[46] and

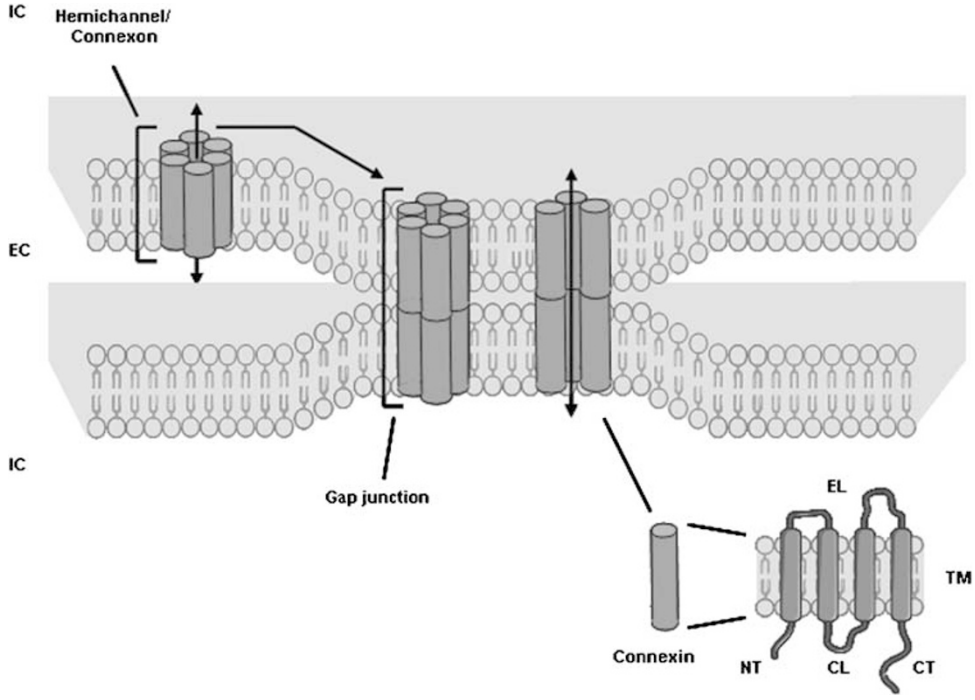

Figure 1 Molecular architecture of gap junctions. GJs are grouped in plaques at the membrane surface of two apposed cells, and are composed of twelve Cx proteins, organized as two hexameric HCs. The Cx protein is organized as four membrane-spanning domains (TM1-4), two extracellular loops (EL1 and EL2), one cytoplasmic loop (CL), one cytoplasmic amino tail (NT) and one cytoplasmic carboxy tail (CT) (EC, extracellular; IC, intracellular)

functionally competent GJ channels,[47,48] although some controversy still exists concerning the latter.[49,50] Furthermore, Panx channels are also permeable to small molecules,[51–53] release ATP,[54,55] open in response to mechanical stimulation,[54] and intracellular Ca^{2+},[56] and are blocked by certain Cx-based GJ blockers.[46,53]

Numerous physiological processes are driven by regulatory molecules that pass through GJs and HCs, which are therefore considered as major gatekeepers in the control of tissue homeostasis. Many efforts in this respect have been focused on their roles in the regulation of cellular proliferation and differentiation.[12,15,57] The exploration of Cx- and Panx-related signaling in cell death, however, has only been initiated in recent years, nevertheless reporting some striking observations in this newly arisen research field.[12,45,58,59] In this review, we will focus on the role of Cxs and their channels as both positive and negative modulators of cell death, mainly apoptosis. We additionally demonstrate the involvement of the recently discovered Panxs in cellular responses to insults.

Connexin-Based Gap Junction Channels and Cell Death

A substantial body of evidence indicates a positive correlation between GJIC and apoptotic activity (Figure 2). Indirect data come from the observation that chemical GJ inhibitors, such as carbenoxolone and 18-beta-glycyrrhetinic acid, prevent apoptosis.[60–63] *Vice versa*, tumor promoters, including peroxisome proliferators and phenobarbital which are known to counteract apoptosis, also inhibit GJIC.[64,65] Furthermore, exogenous introduction of Cxs in a plethora of experimental models was found to facilitate apoptotic cell death (Table 1).[30,63,66–78] Various cell death models have demonstrated the clustering of dying cells, indicating the spread of death signals to neighboring cells through GJs.[63,79,80] This phenomenon of 'bystander death' (the 'kiss of death') has gained a great deal of attention for it opens up the possibility to therapeutically limit the wave of secondary injury in the context of stroke or brain trauma,[3,80–84] and to amplify the potency of cancer treatment. With respect to the latter, the 'suicide gene/prodrug therapy' is a well-known model whereby malignant cells are transfected with the *herpes simplex* virus-thymidine kinase (HSV-tk) gene, followed by treatment with the prodrug ganciclovir (GCV). Following phosphorylation to GCV-triphosphate, this cytotoxic compound competitively inhibits the incorporation of endogenous deoxyguanosine triphosphate into the DNA, resulting in the termination of DNA synthesis and the onset of apoptosis.[85,86] In several tumor cell models, cells that lack the suicide gene and that surround a HSV-tk^+ cell are also killed by GCV treatment because of diffusion of GCV-triphosphate through GJs connecting those cells.[85–87] Another form of bystander killing is mediated by the transfer of viral peptides through Cx-related communication. Neijssen *et al*.[88] discovered that a cell expressing viral proteins and its closest neighbors are killed by cytotoxic T-cells, because the adjacent cells receive the viral peptides through GJs. Thus, GJ immunological coupling could mediate the elimination of uninfected bystander cells or those in the earliest phases of infection.

In-depth studies have revealed that Cx-related communication is modified during the cell death process (Table 2). Particularly the early phases of apoptosis require

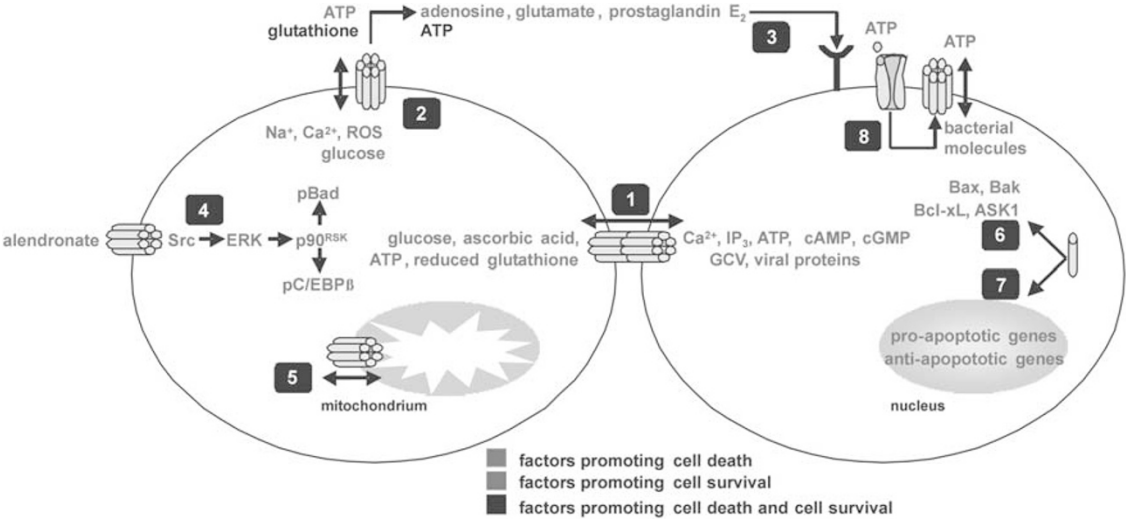

Figure 2 Connexin- and pannexin-related signaling in cell death. Cxs can affect the cell death process through a number of mechanisms, involving GJIC (1), HCs (2–5) and Cx proteins as such (6,7). GJ channels can accommodate direct exchange of cell death and cell survival signals between cells (1). HCs may contribute to cell death by four different mechanisms: by the entry of cell death or the loss of cell survival signals (2), through paracrine signaling of death or survival messengers (3) by HC-mediated transmembrane signal transduction (4) or by affecting mitochondrial functioning (5). Cx proteins as such can associate with cell death regulators (6) or influence the expression of these molecules (7). HCs composed of Panxs may act as a permeabilization pore by itself or as a part of the P_2X_7R death complex (8), allowing ATP to leave the cell or bacterial molecules to make their way into the cell. Although solid scientific data are currently not available, both processes might contribute to cell death. The figure is based on references mentioned in the text. It should be noted that many of the first and second messengers depicted are not cell death or survival messengers *per se*, but rather substances that may lead to cell death or survival under specific conditions that are discussed in the text. (ASK1, apoptosis signal-regulating kinase 1; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; ERK, extracellular signal-regulated kinase; GCV, ganciclovir; IP_3, inositol trisphosphate; pBad, phosphorylated Bad; pC/EBPβ, phosphorylated CCAAT/enhancer-binding protein β; ROS, reactive oxygen species)

Table 1 Interfering with connexin gene expression influences cell death

Model[a]	Cell death	Evaluation method	Mechanism	Reference
Nervous tissue – primary cultures and tissues				
Cx43-deficient mouse astrocytes	↓[b]	Dextran-LY uptake LDH release		24
Heterozygote Cx43$^{+/-}$ mouse astrocytes	↑[b,c]	TUNEL assay	Induced caspase-3 activation	103,116
Cx43-deficient mouse astrocytes		Caspase-3 staining		
Cx43-silenced primary rat astrocytes	↑[b,c]	Hoechst staining		106
Cx43-silenced rat optic nerve segments	↓[b]	PI staining		160
Rat hippocampal organotypic slice cultures Cx26 and Cx32-silenced Cx43-silenced Cx43 KO mice	↓[b]	PI staining		83
Cx32-deficient mouse oligodendrocyte progenitor cells	↑[c]	TUNEL assay		111
Cx36-deficient primary rat hypothalamic cultures	↓[b,c]	Calcein-AM staining		61
Nervous tissue – cell lines				
Cx43-transfected mouse N2A neuroblastoma cells	↑[b,c]	Calcein-AM/EB staining	Uptake of ROS	30
Cx43-transfected rat C6 glioma cells (C6Cx43)	↑[b,c]	Caspase staining Annexin V assay		63
Cx43-silenced C6Cx43 cells	↓[b,c]	Hoechst 33342 staining EB staining		
Cx43-transfected human U251 glioblastoma cells	↑[b,c,d]	TUNEL assay Hoechst staining	Reduced Bcl-2 expression No effect on Bax-1, Bad-1, Bcl-x_L, Mcl-1 expressions	68,69
Cx43- and Bcl-2-transfected rat C6 glioma cells in co-culture	↑[b,c]	Annexin V assay TUNEL assay Hoechst staining		81
Cx43-transfected rat C6 glioma cells	↓[b,c]	Annexin V assay Caspase-3, -8 assay TUNEL assay	Reduced caspase-3 activation No effect on caspase-8 activation	106
Transfected rat C6 glioma cells		TUNEL assay		110
Cx43	↓[b,c,d,e]	Hoechst staining		
Cx40	↓[b,c,d,e]	Alamar blue assay		
Cx32	↓[b,c,d,e]			
Cx37-transfected mouse N2A neuroblastoma cells	No effect[c]	XTT assay		74
Other tissues – primary cultures and tissues				
Cx43 dominant negative mutant-transfected chick leg bud mesenchymal cells	↑[b,c]	FACS analysis		108
Cx43-deficient mouse embryonic heart tissue	↑[c]	TUNEL assay		114
Cx43 antisense oligonucleotide-treated rat neonatal ventricular myocytes	↑[c]	TUNEL assay Hoechst staining		115
Cx32-mutant-transfected Xenopus laevis oocytes	↑[d]	Morphological		36
Cx26-mutant-transfected Xenopus laevis oocytes	↑[d]	Morphological		37
Cx26-inactivated mouse inner ear epithelial cells	↑[c]	TUNEL assay Caspase-3 staining	Induced caspase-3 activation	105
Cx32- and Cx26-deficient mouse liver tissue	↑[c]	TUNEL assay		112
Transfected human umbilical vein endothelial cells		XTT assay	Induced caspase-3 activation	74
Cx37	↑[c]	TUNEL assay		
Cx40	No effect[c]	PI staining		
Cx43	No effect[c]	Caspase-3 assay Annexin V assay Morphological		
Cx45-deficient mouse vascular tissue	↑[c]	TUNEL assay		109
Other tissues – cell lines				
Cx43-silenced rat MC lung epithelial cells	↓[b,c]	Yo-Pro staining Hoechst 33342 staining PI staining	Uptake of ROS	30
Cx43-transfected human HeLa cervical carcinoma cells	↑[b,c,f]	AO/EB staining		70
Cx43-transfected human LNCaP prostate cancer cells	↑[b,c]	TUNEL assay Annexin V assay PI staining Caspase-8 assay	Induced caspase-8 activation	78
Cx43 dominant negative mutant-transfected rat BC31 bladder carcinoma cells	↓[c]	FACS analysis EB staining PI staining		80
Cx43-transfected HeLa cells	↑[b,c,d,f]	Annexin V assay FACS analysis Hoechst 33258 staining Nucleosomal DNA fragmentation PARP fragmentation Caspase-3 assay		137

Table 1 *(Continued)*

Model[a]	Cell death	Evaluation method	Mechanism	Reference
Cx32-transfected human Caki-2 renal carcinoma cells	↑[c]	DNA fragmentation Caspase-3 assay	Induced caspase-3 activation Reduced Bcl-2, Bcl-x_L expression No effect on Bax expression	66,67,177
Cx32-transfected human HeLa cervical carcinoma cells	↑[b,c,f]	AO/EB staining		70
Cx32-transfected human A549 lung adenocarcinoma cells	↑[b,c]	PI staining FACS analysis		72
Cx32-transfected baby hamster kidney cells	↑[b,c]	TUNEL assay Caspase-3, -6, -8, -9 staining Annexin V assay	Induced activation of caspase-3, -6, -9 No effect on caspase-8 activation	77
Cx26-mutant-transfected human embryonic kidney (HEK) 293 cells	↑[c,d]	TUNEL assay		34
Cx26-transfected human PLC/PRF/5 hepatoma cells	↑[c]	ssDNA staining		71
Cx26-transfected human T24, UM-UC-3, UM-UC-6, UM-UC-14 bladder cancer cells	↑[c]	TUNEL assay FACS analysis	Reduced Bcl-2 expression	75
Cx26-transfected human LNCaP, PC-3, DU-145 prostate cancer cells	↑[c]	FACS analysis	Reduced Bcl-2 expression	76
Cx37-transfected rat NRK kidney epithelial cells	No effect[c]	XTT assay		74

AO, acridine orange; EB, ethidium bromide; FACS, fluorescence-activated cell sorting; LDH, lactate dehydrogenase; LY, lucifer yellow; PARP, poly-ADP-ribose polymerase; PI, propidium iodide; ROS, reactive oxygen species; ssDNA, single-stranded DNA; TUNEL, terminal dUTP nick end labelling. [a]A distinction is made between nervous tissues and tissues/cells from other sources. Studies are further categorized according to the Cx species of which the expression is influenced. [b]Exogenously induced cell death. [c]Apoptosis-related cell death. [d]Effect on cell death is not related to gap junction function. [e]Necrosis-related cell death. [f]Apoptotic necrosis (secondary necrosis).

GJIC,[62,70,89–91] but with progression of cell death, GJIC drastically declines culminating in the absence of cell coupling between apoptotic bodies. The abolishment of GJIC thus coincides with the typical morphological transformations that occur in apoptosis and results from an increased removal of GJ channels from the PM and not from their functional closure.[70] In a recent study, Theiss *et al*.[92] exposed primary bovine lens epithelial cells and mouse NIH-3T3 fibroblasts to a number of well-established apoptosis-inducing agents. The authors noticed a decrease in GJIC that was associated with caspase 3-mediated degradation of Cx43. Similarly, activation of apoptosis in primary cultures of chick lenses resulted in caspase 3-mediated truncation of Cx45.6, a reaction that was inhibited by casein kinase 2-mediated phosphorylation of Cx45.6.[93] As a matter of fact, phosphorylation is likely to be a major mechanism responsible for GJIC alterations during apoptosis (Table 2). In rabbit lens epithelial cells, H_2O_2 induced caspase 3-dependent apoptosis that was associated with activation of protein kinase Cγ, the phosphorylation of Cx43 and Cx50, and the disassembly of GJ plaques abolishing GJIC.[94,95] Induction of apoptosis in rat liver epithelial WB-F344 cells by the hydrophobic platinum IV complex LA-12 was also linked to suppression of GJIC and the disappearance of Cx clusters from the cell membrane surface. LA-12 thereby triggered rapid Cx43 hyperphosphorylation mediated by the mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (MEK/ERK) pathway.[96] Increased Cx phosphorylation does not always go hand in hand with a loss of GJIC. Indeed, in the WB-F344 rat liver epithelial cell model, serum deprivation[91] and exposure to the histone deacetylase inhibitor suberoylanilide hydroxamic acid[97] enhanced GJIC, particularly during the early phases of apoptosis, and simultaneously increased Cx43 phosphorylation. Inversely, inhibition of GJIC in primary bovine lens epithelial cells and primary human corneal fibroblasts by staurosporine[92] and TNFα[98] was accompanied by a decrease in the Cx43 phosphorylation status. Of note, alterations in Cx phosphorylation are frequently allied with modifications in cellular localization. The drastic decrease in GJIC that is observed during mitosis, for instance, results from the cytoplasmic redistribution of a unique Cx43 phosphoform called 'Cx43m' or 'Cx43P3'.[99–101] The latter is generated by the cyclin-dependent kinase 1/cyclin B1 complex, which not only controls the G2/M transition of the cell cycle, but also plays a crucial role in the initiation of apoptosis.[91,99–101] In fact, induction of apoptosis by choline deficiency increased Cx43 immunoreactivity in the cytoplasm of rat liver epithelial cells. Restoration of the Cx43 membrane localization, and consequently of GJIC, as well as enhanced cell survival was brought about by 8-bromo-cAMP,[102] a well-known inductor of Cx43 phoshorylation.[22]

Contradictory to the paradigm that GJIC facilitates apoptosis are a number of investigations showing that GJs may impede the occurrence of cell death (Figure 2).[103–116] In line with this notion are a number of reports describing induction of apoptosis by chemical GJIC inhibitors[107,108,117–119] as well as by Cx mimetic peptides,[120] which are peptides identical to a short Cx sequence that act as GJ blockers.[121] Healthy cells may indeed provide their dying neighbors with rescue signals[3] or cells in danger may dilute toxic substances toward healthy neighbors through GJs.[3,103] This event whereby cell survival is promoted has been designated the 'good Samaritan' effect or the 'kiss of life', as opposed to the 'bystander death' phenomenon.[85] Thus, there seems to be a clear two-way traffic between (early) apoptotic and non-apoptotic cells[80] and, depending on the status and environmental context of the cell that receives the signal, two opposite biological outcomes can be achieved, namely cell death or cell survival.[3]

Table 2 Connexin-related modifications during cell death

Model	Cell death induction	Observation	Reference
Rat hippocampal slices	OGD[a]	Induced GJIC Induced Cx36 expression	60
Rat primary hypothalamic cultures	Different modes of NMDA receptor-regulated cell death[a]	GJIC, but no HC involved	61
Rat MC mammary tumor cells Rat L2 lung epithelial cells Cx43-transfected mouse N2A neuroblastoma cells	H_2O_2[a] Cigarette smoke extract[a]	HC activation	30
Cx43- or Cx32-transfected human HeLa cervical carcinoma cells	Cycloheximide[a] Etoposide[a] Taxol[a] *Cis*-platinum[a] Camptothecin[a]	GJIC present in early cell death phases Reduced GJIC during cell death progression, associated with gap junction internalization, but not with closure of gap junctions HC functioning decreased during the development of apoptosis	70
Rat BC31 bladder carcinoma cells	Spontaneous[a]	GJIC present between apoptotic and non-apoptotic cells No difference in Cx43 expression between apoptotic and non-apoptotic cells	80
Rat hippocampal organotypic slice cultures	Weight-drop injury	Transient potentiation of GJIC after injury	83
Primary quail granulosa cells	Serum deprivation[a]	Induced GJIC Reduced Cx43 expression	89
Rat WB-F344 liver epithelial cells	Serum deprivation[a]	Induced GJIC in early cell death phases, associated with induced Cx43 expression/phosphorylation Reduced GJIC during cell death progression	91
Primary bovine lens epithelial cells Mouse embryonic NIH 3T3 fibroblasts	Staurosporine[a] Etoposide[a] Puromycin[a] Cycloheximide[a]	Reduced GJIC Reduced Cx43 expression/phosphorylation	92
Rat WB-F344 liver epithelial cells	LA-12[a]	Reduced GJIC Induced Cx43 phosphorylation	96
Rat WB-F344 liver epithelial cells (*ras*-transformed)	Suberoylanilide hydroxamic acid[a]	Induced GJIC Induced Cx43 expression/phosphorylation	97
Human corneal fibroblasts	TNFα[a]	Reduced Cx43 translation and phosphorylation No effect on Cx43 transcription Reduced GJIC	98
Rat WB liver epithelial cells Human Hep3B liver epithelial cells	Choline depletion[a]	Reduced GJIC, associated with cytosolic Cx43 redistribution No effect on Cx43 expression	102
Chick leg bud mesenchymal cells	TGF-β_3[a]	Reduced Cx43 expression	108
Xenopus laevis oocytes	Cytochrome C[a]	Induced GJIC	122
Primary rat astrocytes	Antimycin A and iodoacetic acid-induced metabolic inhibition	HC opening Reduced GJIC Dephosphorylation of Cx43	32
Primary mouse astrocytes	Antimycin A and iodoacetic acid-induced metabolic inhibition	HC opening Increased surface Cx43 expression Dephosphorylation/nitrosylation of Cx43	31
Cx26-mutant-transfected human embryonic kidney (HEK) 293 cells	Spontaneous[a]	Cx26-HC opening	34
Cx32-mutant-transfected Xenopus laevis oocytes	Spontaneous	Cx32-HC opening	36
Cx26-mutant-transfected Xenopus laevis oocytes	Spontaneous	Cx26-HC opening	37
Cx43-transfected human HeLa cervical carcinoma cells	Staurosporine[a]	Cx43-HC opening Increased surface Cx43 expression/dephosphorylation	137
Primary human renal proximal tubule cells	ATP-depletion induced by glycolysis inhibition[b]	Cx43-HC activation, associated with disrupted ATP-flux Dephosporylation of Cx43 as possible mechanism	141
Rat spinal cord	SCI by cutting into segments	Induced Cx43 expression	159
Rat optic nerve segments	OGD	Increased Cx43 expression	160
Human MG63 osteosarcoma cells	TNFα[a]	Reduced Cx43 translation	189
Human osteoblasts	TRAIL[a]	Induced Cx43 transcription Reduced GJIC	
Human MG63 osteosarcoma cells Human osteoblasts	Serum deprivation[a]	Reduced Cx43 translation, associated with cytosolic Cx43 redistribution Induced Cx43 transcription Increased GJIC	189

ATP, adenosine triphosphate; Cx, connexin; HC, hemichannel; GJIC, gap junctional intercellular communication; JNK, c-Jun N-terminal-kinase; LPS, lipopolysaccharide; MKK7D, mitogen-activated protein kinase kinase 7D; NAD, nicotine adenosine dinucleotide; NMDA, N-methyl D-aspartate; OGD, oxygen/glucose deprivation; SCI, spinal cord injury; TGF-β, transforming growth factor β; TNFα, tumor necrosis factor α; TRAIL, TNF-related apoptosis-inducing ligand. [a]Apoptosis-related cell death. [b]Necrosis-related cell death.

The biochemical nature of the signals that actually mediate these effects, in particular those that propagate the cell death message, are largely unknown. Ca^{2+} has been proposed as a 'master' cell death messenger (Figure 2). The onset of apoptosis is indeed frequently accompanied by drastic alterations in cytoplasmic Ca^{2+} concentration, and several crucial apoptosis effectors are known to depend on Ca^{2+}.[3,58,63,80,89,122] However, Ca^{2+} diffusion through GJs

may be limited by its interactions with more slowly mobile Ca^{2+}-binding proteins. The Ca^{2+} messenger IP_3, produced by phospholipase C activation and triggering Ca^{2+} release from the endoplasmic reticulum (ER), is probably a better suited candidate as it can pass through GJs,[123] its ER IP_3-receptor is modulated by Bcl-2[124,125] and the ensuing Ca^{2+} changes may trigger cytochrome *C* (CytC) release from mitochondria.[126] Released CytC on its turn can interact with IP_3 receptors to potentiate ER Ca^{2+} release and to exacerbate mitochondrial Ca^{2+}-driven CytC release in a vicious circle.[127] Recent work further suggests that the pattern of Ca^{2+} changes – steady or oscillatory changes – may determine the outcome towards either cell death (sustained Ca^{2+} change) or survival (Ca^{2+} oscillations).[128] The signature of the Ca^{2+} signal is largely influenced by the level of IP_3 and the sensitivity of its receptor.[129] As a consequence, the modulation of IP_3 receptors by Bcl-2 family proteins[125] will determine the Ca^{2+} load of mitochondria and will thus be expected to be decisive in initiating apoptosis through mitochondrial CytC release. CytC has too high a molecular weight (12.4 kDa) to pass as a death messenger through GJs or HCs, so IP_3 remains as a very likely candidate to communicate the cell death message. Other proposed candidate 'killer messengers' that can pass through GJs include cAMP and cyclic guanosine monophosphate (cGMP).[58,74] Potential 'rescue messengers' on the other hand include the energy molecules glucose and ATP or the free radical scavengers ascorbic acid and reduced glutathione that are all able to flow through GJs and favor cell survival.[3] GJ channels display selective permeability towards the proposed messengers and this typically depends on their Cx composition.[15–19] Seul *et al*.[74] found that adenoviral delivery of Cx37, but not of the other vascular Cx40 and Cx43, induces apoptotic cell death in human umbilical vein endothelial cells. This effect was cell- and species-specific as Cx37 did not trigger cell death in mouse N2A neuroblastoma cells and rat NRK kidney epithelial cells. These findings demonstrate that conveying a cell death or survival response through GJ channels is a selective and well-coordinated process, involving specific Cxs and biochemical messengers rather than just the movement of common noxious or vital molecules between cells.

Connexin-Based Hemichannels and Cell Death

For a long time, HCs were classified as mere structural precursors of GJs. A large body of recent evidence, however, suggests that they must be considered as functional channels controlled by various extracellular and intracellular signals, thereby providing a unique pathway for transport and signaling. Of note, many of the signals that trigger HC opening, or responses attributed to HCs, are also involved in the signaling cascades leading to cell death,[11] including cytoplasmic Ca^{2+} changes and oxidation or nitrosylation reactions. Being a pore permeable to quite a large range of substances, HC opening may furthermore help in setting off cell death by causing membrane depolarization, the collapse of ionic gradients, loss of small metabolites and elevation of cytoplasmic Ca^{2+}.[130]

As holds for GJs, cell death may have a profound influence on the status of the HCs (Table 2). This has been well exemplified in the case of ischemia-related cell injury where the involvement of HCs as 'pathogenic pores' has been demonstrated.[3,33] Indeed, metabolic inhibition of astrocytes was found to accelerate cell death, which was directly associated with Cx43 HC opening. The molecular mechanism by which it activates HCs is, however, not entirely known, but may involve dephosphorylation and/or oxidation.[31,32] With respect to the latter, Retamal *et al*.[31] showed that nitrosylation of intracellular Cx43 cysteine residues by nitric oxide (NO), a protein modification likely to occur under oxidative stress, could be at the heart of HC opening. Along the same line, NO was found to mediate Cx43 HC activation in astrocytes under proinflammatory conditions.[131] The importance of oxidative stress as a key trigger is underscored by the observation that cigarette smoke extract and H_2O_2 may open HCs by depolarizing the cells, thereby facilitating ROS entry into the cell.[30] HC function may also be drastically affected by genetic factors. Mutations in Cx32, Cx30 and Cx26 genes result in hereditary peripheral neuropathy, hidrotic ectodermal dysplasia, and nonsyndromic hereditary hearing impairments and skin disease, respectively. In all cases, the phenotype is caused by abnormal HC opening which could adversely affect cell viability.[34–37,132,133] Gomez-Hernandez *et al*.[35] revealed that HC dysregulation in inherited peripheral neuropathy, caused by a Cx32 mutation, is due to alterations in a Ca^{2+}-binding site that interacts with extracellular Ca^{2+} to keep the HCs closed under physiological conditions. A similar mechanism could account for aberrant HC gating and cell death caused by a Cx26 mutation located right next to this putative Ca^{2+}-binding site, a mutation that is known to lead to nonsyndromic hereditary deafness.[34]

Overall, there are at least four possible mechanisms by which HCs could contribute to cell death or survival (Figure 2). First, the bidirectionally permeable HC pathway may facilitate the uptake of toxic or survival-enhancing substances, and may contribute to the loss of essential metabolites.[30,38,59,70,134–137] As well-documented ATP release channels, HCs may lower the intracellular ATP content and play as such a decisive role in the balance between cell death through necrosis or apoptosis.[63,70,138] ATP is also necessary for the phosphorylation of Cxs which, on its turn, influences HC gating.[28,32,139–141] In Shigella infection, hemichannel ATP release may promote bacterial invasion and dissemination.[142] Another compound released through HCs is glutathione.[43] The loss of intracellular glutathione may compromise the antioxidant defense at the cells' inside but its consequent accumulation outside the cells may as well act beneficially as it can intercept in a more direct manner invading free radicals threatening the cell.[43] Another potentially beneficial role of Cx43 HCs is suggested from experiments in astrocytes that demonstrated increased open probability of Cx43 HCs and glucose uptake in response to exposure to proinflammatory cytokines.[131]

A second level where HCs can influence the balance between cell death and survival involves a paracrine communication component. The release of metabolites such as ATP, glutamate or prostaglandins through HCs combined with their extracellular diffusion and action (directly or through break-

down products like adenosine)[143,144] on corresponding receptors on remotely located cells may exert toxic as well as survival effects.[145–151] Ligand-receptor binding of these messengers is often accompanied by a rise in cytoplasmic Ca^{2+},[126,145,152–154] that on its turn may trigger a new cycle of HC responses or is even influenced by HC activity.[26,35,38,126,152] In a model of cochlear explant cultures, the local infliction of a cell-damaging stimulus caused a spatiotemporal spread of cell death in hair cells that was related to ERK1/2 activation, dependent on the associated intercellular Ca^{2+} wave and ATP release, and inhibited by carbenoxolone.[155] Once released, ATP is rapidly degraded to adenosine which displays both neuroprotective and -toxic properties.[143,144] Combined *in vitro* and *in vivo* studies have demonstrated that ischemic pre-conditioning in the brain promotes ATP efflux through HCs and thereby protects against a second exposure to ischemic conditions through adenosine.[156] Another messenger released through HCs is glutamate, a well-documented paracrine messenger of cell death in the brain. The presence of this substance, released by HCs, in conditioned media from microglia and macrophages treated with proinflammatory molecules resulted in marked neuronal cell death when neurons were exposed to it.[136,157] Conditioned media from activated microglia also affected astrocytes as it reduced their GJIC and induced HC opening.[131] The latter can have a drastic influence on neuronal survival as well, as the capacity of 'spatial buffering', which is provided by coupling of astrocytes through GJ channels and is essential for the control of the composition of the extracellular environment, decreases.[3,158] This is observed in an *ex vivo* model for spinal cord injury where Cx43 channels appeared to play a dual role; HCs seemed to be involved in cell swelling and the spread of neurotoxins early after injury, whereas GJs were required for spatial buffering between astrocytes and long-term survival of neurons.[159] A paracrine signaling component may not only be of importance for the expansion of cell death or survival but could also assist in the recruitment of both innate and adaptive components of the inflammatory response system.[160] Certain types of dead cells can trigger inflammation by release of danger signals or 'damage-associated molecular patterns' that are normally located inside the cells.[161,162] At a more speculative level, it is conceivable that these endogenous danger signals (e.g., uric acid, 168 Da) may be shared through GJs with neighboring cells and released through HCs.

A third cell survival/cell death regulatory platform includes HCs as a transmembrane signal transduction pathway. The prototypic example here is the involvement of HCs in the anti-apoptotic actions of alendronate, a drug used to counteract osteoporosis. Alendronate induces the opening of Cx43 HCs in dexamethasone-treated osteoblasts and osteocytes, which activates Src kinase and ERK. Subsequent phosphorylation by the p90 ribosomal S6 kinase ($p90^{RSK}$) leads to an inactivation of the cytoplasmic pro-apoptotic Bcl-2 protein Bad and binding of pro-caspases by the phosphorylated CCAAT/enhancer-binding protein β (pC/EBPβ), both favoring survival of osteoblasts and osteocytes.[163]

A fourth possible link between HCs and cell survival/cell death relates to modifications in the subcellular localization of HCs. Upon ischemic pre-conditioning, a form of cardioprotection, Cx43 translocates from the PM to the inner mitochondrial membrane through a heat-shock protein 90-dependent pathway.[164–166] The functional relevance of mitochondrial Cx43, however, is not clear. It has been postulated that Cx43 is part of a multiprotein complex that somehow controls mitochondrial homeostasis.[117] Mitochondrial Cx43 could also form HCs that serve as a conduit for ion fluxes,[59,165–167] a function reminiscent of the apoptosis-regulating Bcl-2 family proteins.[117] Mitochondrial Cx43 was recently found to be a major regulator of cardiomyocyte apoptosis.[117]

Non-Channel Aspects of Connexins in Relation to Cell Death

Several studies have shown that Cx proteins as such can influence tissue homeostasis independent of their channel-forming capabilities. In most cases, the evidence is based on experiments in which Cx expression was artificially modified (Table 1). Cx overexpression was found to trigger cell cycle arrest[168–173] and cellular differentiation[174] without influencing Cx channel activity in any way. In a similar manner, transfection of C6 rat glioma cells with Cx43, Cx40 or Cx32 genes resulted in an increased resistance to exogenously induced cellular injury, independently of GJIC.[110] Forced expression of Cx43 in U251 human glioblastoma cells, on the other hand, did not increase GJ coupling but enhanced the apoptotic response to chemotherapeutics.[69] Furthermore, research in a model of apoptosis-primed primary quail granulosa cells showed that Cx43 expression was inversely related to both the apoptotic index and the level of GJIC, suggesting distinct roles for GJIC and Cx43 in cell death and cell survival, respectively.[89]

The mechanisms that underlie non-channel Cx protein contributions to cell death remain to be elucidated (Figure 2). It has been suggested that Cxs participate in cell death pathways through direct interaction with apoptotic factors. The cytoplasmic co-localization of Cx26 and Cx43 with the Bcl-2 proteins Bak, Bcl-xL and Bax, in human breast cancer cells and human colorectal cancer cells, respectively, could be in favor of this idea.[175,176] More recently, Cx43 was reported to directly interact with apoptosis signal-regulating kinase 1 (ASK1).[106] Cxs may also be involved in the control of cell death-related gene expression. A number of papers have reported changes in the expression of individual subsets of apoptotic factors while interfering with Cx expression.[67–69,72,75,76,168,177] Microarray analysis of heart tissue from Cx43 knockout mice showed altered expression of a wide spectrum of apoptotic genes, including Bok, Bax, Bid, Diablo, caspase 6 and caspase 9.[114] Iacobas *et al.*[178] performed a similar in-depth study, by analyzing the transcriptome of Cx43-null astrocytes in mice. Interestingly, they showed that both pro-apoptotic (e.g., Map4k) and anti-apoptotic (e.g., Bcl-xL) genes are affected, a finding that might explain the often contradictory reports, associating both induction and inhibition of apoptotic cell death with the presence of Cxs. The exact nature of the link between Cxs and apoptosis-related gene expression is still a matter of debate. An attractive hypothesis is that Cxs are directly involved in transcriptional control. In this respect, 'Cx response elements' have been proposed to modulate gene expression in rat osteocytes.[179,180] An active role for Cxs in the

regulation of gene expression is further supported by the observation that (transfected) Cxs, or at least specific parts of these molecules (i.e. the C-terminal region), can reside in the nuclear compartment.[170,181,182] The latter may, however, also be a result of the well-known interaction of Cxs with transcriptional regulators and/or mediators of crucial signaling pathways. A broad range of Cx-binding partners have actually been characterized, some of which are acknowledged regulators of gene expression.[183,184] Cx43, for instance, interacts with β-catenin, a key player in Wnt signaling.[185] In the cell nucleus, β-catenin forms a complex with the T-cell factor that facilitates the transcription of a number of target genes that are relevant to apoptosis, including survivin,[186] Bcl-xL[187] and Bcl-2.[188] Cx43 expression itself is also controlled by Wnt signaling.[185] In fact, TNFα and TRAIL not only increase Cx43 degradation but also augment the Cx43 mRNA content in human osteosarcoma cells. The enhanced Cx43 gene transcription is thought to represent a reflexive response to apoptosis and is likely to be mediated by β-catenin.[189]

Pannexins and Cell Death

The structural and functional similarities between Cxs and Panxs raise the possibility that Panxs might be implicated in cell responses to several pathological insults as well, for example ischemic neuronal cell death. Exposure of pyramidal neurons from cortex or hippocampus to oxygen and glucose deprivation (OGD), a condition that mimics some aspects of ischemia, triggered opening of a large conductance and the bidirectional transfer of small fluorescent tracer molecules.[52] Long OGD exposures resulted in irreversible current activation, neuronal swelling and membrane breakdown. Based on the absence of Cxs but presence of Panx1 in these neurons, the large single channel conductance and the effect of the (non-specific) blockers carbenoxolone and the HC-inhibiting La^{3+} ions, the authors concluded that these responses are likely to be mediated by Panx1 HCs. The mechanism by which OGD leads to Panx HC opening is not clear but may involve an increase in cytoplasmic Ca^{2+}, which is known to occur in hippocampal neurons after ischemic injury and to activate Panx HCs.[56,152,153] The proposed mechanism of dephosphorylation-induced opening of Cx43 HCs in metabolically inhibited astrocytes could also account for Panx HC opening as multiple phosphorylation sites have been predicted in both the cytoplasmic loop and the C-terminal tail of the Panx protein.[45] There is, however, no clear evidence yet of functional modulation of Panx HCs by this covalent modification.

Similar to Cxs, Panx1 was found to be critical for inflammatory responses in lipopolysaccharide-stimulated macrophages pulsed with ATP[53] or exposed to the pore-forming toxins nigericin and maitotoxin,[190] through HC-dependent or HC-independent mechanisms respectively. With respect to the former, studies further demonstrated that Panx1 HC opening can be induced by ATP stimulation of P_2X_7 receptors (P_2X_7R) and thereby provides an entry pathway for bacterial inflammatory proteins to make their way to the cytosol.[191] P_2X receptors are a family of ionotropic receptors that open non-selective cation channels when activated by pyrimidine nucleotides. Interestingly, during prolonged agonist exposure, certain of these receptors like the P_2X_7R lead to the opening of a non-selective pore capable of passing large hydrophilic molecules, leading to cell death by apoptosis or necrosis.[147,148,192–194] In fact, many of the characteristics of the P_2X_7R-activated pore seem to match those of HCs, including blockade by prototypical GJ channel blockers.[195] A number of studies have demonstrated that the large permeabilization pore may correspond to Panx1 HCs being recruited by prolonged P_2X_7R activation.[53,190,193,196] Binding of the tyrosine kinase Src to the P_2X_7R seems to be a crucial step in this process.[196] In support of this finding is the observation that prolonged stimulation of the P_2X_7R by ATP or an agonist induced cell death in oocytes and human astrocytoma cells, a process that was affected by carbenoxolone. Thus, Panx1 appears to be the molecular substrate for the permeabilization pore or 'death receptor channel' recruited into the P_2X_7R-signaling complex.[193] Panx1 HCs can also be activated by ATP through the metabotropic P_2Y_1 and P_2Y_2 receptors, presumably through increases in cytoplasmic Ca^{2+}.[56] This, however, is not accompanied by subsequent membrane permeabilization and is therefore thought to serve more physiological functions, such as the communication of Ca^{2+} signals between cells,[55] a communication pathway that may be further supported by Panx1 GJs connecting the cells.[48,55,56] In addition, a recent study suggests that Panx1 may also be present in the ER, forming Ca^{2+}-permeable channels and representing one of the mechanisms responsible for ER Ca^{2+} leak.[48] The latter may on its turn trigger apoptotic cell death by facilitating Ca^{2+}-induced opening of the mitochondrial permeability transition pore, which has been implicated in the release of CytC from mitochondria.[126,197]

In contrast to Panx1, a neuroprotective role was proposed for Panx2. *De novo* and transient expression of Panx2 in astrocytes was observed in the brain of adult rats following ischemia-reperfusion and in co-cultures of hippocampal neurons and astrocytes. The authors hypothesized that Panx2 mediates its neuroprotective effect through the formation of HCs allowing the release of signaling molecules devoted to influence the cellular metabolism and the redox status of the surrounding environment.[198]

Conclusions and Perspectives

The maintenance of the homeostatic balance is controlled by the global interplay between three major communicative networks that make use of extracellular, intracellular and intercellular signaling pathways. Direct intercellular communication is mainly mediated by GJ channels composed of Cxs. The key roles of GJs in the control of cellular proliferation and differentiation have been demonstrated at numerous occasions over the last few decades.[12,15,57] Their participation as mediators of cell death communication, particularly of apoptotic cell death, has only gained interest in recent years but has already brought up the important insight that not only GJs but also their structural precursors, the HCs and the Cx proteins as such, influence the cell death process. Strikingly, Cx-related communication has been found to both facilitate or counteract the cell death process. Such opposed functions may, to some extent, relate to differences in the model

systems used, including the mode of cell death induction. Different cell death activators may indeed recruit distinct signaling cascades and messengers that may or may not be able to pass or activate Cx channels. An additional factor that may complicate the situation is the fact that the two channel types, although composed of the same protein, are differentially regulated. Under normal conditions, GJs are open and HCs are closed and two recent papers indicate that these two channels can be differentially influenced by the same stimulus,[28,131] for example by arachidonic acid which inhibits GJs and potentiates HC responses. The decision to stay alive or die may further depend on the condition of the cell receiving the message carried through Cx channels. Thus, if the concerned cell is concomitantly exposed to stress stimuli, then Cx-related communication may switch from a survival program to a death mode. Furthermore, the contributions of Cxs to cell death communication may be adjoined by the Panx channel family. The specifics and possible differences in the regulation of Cx *versus* Panx channels are currently largely unknown and a major challenge for future work is to elucidate the exact contribution of GJs and HCs composed of either Cxs or Panxs, to cell death processes of both the apoptotic or non-apoptotic type. An equally important challenge concerns the development of specific blockers that affect HCs or GJs only. Comparisons between Cx-expressing cells and cells treated with protocols to silence the concerned gene or obtained from knockout animals are necessary to unequivocally determine the involvement of Cx HCs.[195] Knockout animal models can, however, not be used to establish the involvement of Cx HCs *in vivo* because both GJs and HCs are suppressed in that case. The quest is still open but progress is, as expected from channels composed of the same building blocks, not in immediate sight. The molecular principles to distinguish GJs from HCs are still hazy. For instance, Cx mimetic peptides composed of a short stretch from one of the extracellular Cx loops are actually expected to open the HCs by a process similar to 'loop gating' but these substances turn out to inhibit the responses attributed to HCs. Clearly, further work is needed to bring progress in this complex but tantalizing field: two distinct channels formed by a single protein that in addition (and beyond its channel function) has intrinsic signaling functions is not a simple case at all! In summary, Cxs and Panxs as well as their corresponding channels represent multilevel platforms for cell death-related signaling. It is expected that, upon the introduction of appropriate experimental tools, more insight will be gained into the exact molecular mechanisms that underlie Cx- and Panx-inherent communication in (apoptotic) cell death.

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