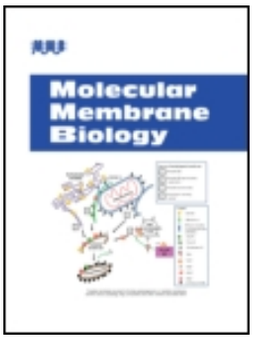

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Gap junctions: structure and function (Review)

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Summary

Gap junctions are plasma membrane spatial microdomains constructed of assemblies of channel proteins called connexins in vertebrates and innexins in invertebrates. The channels provide direct intercellular communication pathways allowing rapid exchange of ions and metabolites up to ~1 kD in size. Approximately 20 connexins are identified in the human or mouse genome, and orthologues are increasingly characterized in other vertebrates. Most cell types express multiple connexin isoforms, making likely the construction of a spectrum of heteromeric hemichannels and heterotypic gap junctions that could provide a structural basis for the charge and size selectivity of these intercellular channels. The precise nature of the potential signalling information traversing junctions in physiologically defined situations remains elusive, but extensive progress has been made in elucidating how connexins are assembled into gap junctions. Also, participation of gap junction hemichannels in the propagation of calcium waves via an extracellular purinergic pathway is emerging. Connexin mutations have been identified in a number of genetically inherited channel communication-opathies. These are detected in connexin 32 in Charcot Marie Tooth-X linked disease, in connexins 26 and 30 in deafness and skin diseases, and in connexins 46 and 50 in hereditary cataracts. Biochemical approaches indicate that many of the mutated connexins are mistargeted to gap junctions and/or fail to oligomerize correctly into hemichannels. Genetic ablation approaches are helping to map out a connexin code and point to specific connexins being required for cell growth and differentiation as well as underwriting basic intercellular communication.

Keywords: Connexin, intercellular communication, channelopathies, knockout mice, trafficking pathways.

Introduction and historical perspective

Cells in tissues and organs co-ordinate and summate their activities by communicating directly with each other. A widespread mechanism ensuring intercellular communication operates at regions of cell adhesion where clustering occurs of paired channels buried in the contacting plasma membranes. It has now become generally accepted that these junctions, called gap junctions, provide a regulated pathway linking the cytoplasms of attached cells, and contribute towards ensuring the integration of metabolic activities by setting up networks of directly communicating cell assemblies. For example, cardiac myocytes synchronize their contractions by communicating electrically across gap junctions dispersed among other adhesive junctions in intercalated discs; the summation of the synchronous beating of individual myocytes accounts for the rhythmic pumping of the heart. Cilia of tracheal epithelial cells are linked by gap junctions that co-ordinate their uni-directional beating to expel fluids and cleanse the pulmonary passages. Smooth muscle cells lining the uterine wall upregulate gap junction numbers to co-ordinate contractions during birth. These overt mechanical examples illustrate the cellular co-ordination underlying tissue/organ functioning enabled by gap junctional intercellular communication. However, 'silent' metabolic and ionic intercellular signalling across gap junctions is constantly ongoing in cell assemblies and is increasingly appreciated to be a key process in development and growth control.

Major progress has been made in illuminating the structural organization, mechanism of assembly, and the functioning of gap junction intercellular communication channels. A number of general reviews cover gap junctional communication (Bennett *et al*. 1991, Goodenough *et al*. 1996, Kumar and Gilula 1996), and books on cardiac gap junctions (Dhein 1998) and brain gap junctions (Spray and Dermietziel 1996) have been published. The present review confines attention mostly to advances reported from the mid 1990s and sits on a short historical account of the evolution of the concept of gap junctions as major mediators of cellular co-ordination.

The emergence of gap junctions as discrete plasma membrane domains most likely to account for direct cell–cell communication was the outcome of the convergence of a number of independently pursued and, apparently, unrelated studies. Pioneering work in the 1950s on fast excitatory transmission in the crayfish giant fibre system (reviewed by Furshpan and Potter 1968) and in neurons of the lobster cardiac ganglion (Watanabe 1958) indicated that these cells communicated directly via electrical pathways. Also, in the 1950s, it was becoming clear that a rapid electrical mode of intercellular communication operating in Purkinje fibres of mammalian heart could explain the syncytial behaviour of the cardiomyocytes (Weidmann 1969). The detection of low-resistance ionic pathways between mosquito salivary gland cells (Loewenstein 1967) also pointed to a direct electrically mediated mechanism of communication. As recollected by Bennett (1997: p352), 'electrical synapses allow multiple cells to act with nearly the precision of a single cell', but at the time the emerging conclusions contradicted the prevailing view of animal cells as independent entities surrounded by an electrically insulating membrane and communicating solely by release of extracellular messengers. Controversies regarding the relative contributions and importance of chemical synapses and electrical junctions as modes of communication in the nervous system have continued, but with increasing acceptance that both mechanisms of intercellular communication often operate in parallel. Permeation of small tracer dyes between rat neocortical neurons pointed to gap junctional pathways in this region of the brain (Peinado *et al*. 1993). Astrocyte intercellular communication

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has now been shown to involve a major gap junctional component (Giaume and Venance 1998, Cotrina *et al*. 2000) and the presence of gap junctions in spinal cord (Kiehn and Tresch 2002) are examples that re-enforce views that gap junctions fulfil important roles in the nervous system. Also, in the late 1960s, the phenomenon of metabolic co-operation between contacting co-cultures of non-excitable cells that allowed the rescue of cells deficient in thymidine or purine metabolism and involving their direct intercellular transfer was described and shown later to be underwritten by gap junctions (Pitts 1998). This mechanism for sharing genetic ability appeared to be the basis of contact inhibition of growth, and is now being explored as a pathway for direct intercellular transfer via gap junctions of toxic chemicals in cancer therapy (Azzam *et al*. 2001). A significant morphological advance in deducing the membrane basis of potential cell–cell communication pathways used Mauthner cell synapses in goldfish brains, where a striking hexagonal arrangement of apposed channels was observed (Robertson 1963). Other approaches applied to liver tissue strengthened views that these regular sub-unit patterns at cell surfaces were likely candidates for mediating direct communication between attached cells (Revel and Karnovsky 1967). Although the biochemical identification of gap junction proteins would take a further 18 years (Kumar & Gilula 1986, Paul 1986), these earlier morphological approaches contributed to drawing clearer functional distinctions between chemical and electrical junctions and between gap junctions and tight junctions. Gap junctions have now been identified in all tissues except in striated muscle where the cells have fused during development. Even loosely associated aggregates of cells, as in lymph glands (Oviedo-Orta *et al*. 2000) and haemopoietic tissue (Cancelas *et al*. 2000, Rosendaal and Krenacs 2000, Montecino-Rodriguez and Dorschkind 2001), are organized as interacting cellular networks extensively coupled by gap junctions. Indeed, the list of cells not utilising this mode of cellular interaction and signalling is confined to erythrocytes, platelets and sperm.

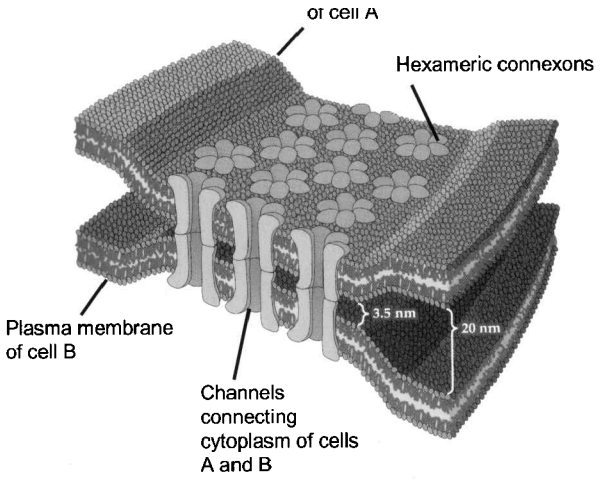

Figure 1. Model of a gap junction. The hexameric connexin subunits in each of the plasma membranes dock to generate the gap junction channel connecting the two cytoplasmic compartments. Reproduced from Purves *et al*. (2001).

Connexins: proteins of vertebrate gap junction channels

2In vertebrates, gap junction channels are constructed of two apposed hexamers of connexin proteins arranged around a central pore (figure 1). Gap junctions appear as plaques of varying size, as these unit channels accrete laterally in the plasma membrane and have been studied by electron microscopy of immunogold labelled freeze fracture replicas (Rash *et al*. 1998), by immunocytochemistry (Severs *et al*. 2001) and by fluorescently tagged connexins (Bukauskas *et al*. 2000, Falk 2000, Rutz and Hulser 2001). Around 20 highly homologous products of connexin genes have been identified in humans and in mice (Willecke *et al*. 2002). Orthologues, with high sequence identity, are increasingly studied in other species, especially Xenopus, chick and zebrafish. Two major connexin classes, *viz*. α and β, and a minor γ-class are noted but human Cx25, Cx30.2, Cx36, Cx40.1 and Cx58 and mouse Cx29, Cx36 and Cx39 are, to date, unclassified (table 1). The major connexin classes appear to have evolved by gene duplication from ancestral genes (Bennett *et al*. 1994). Since most cell types express more than one connexin isoform, the hexameric connexin hemichannels can be homomeric or heteromeric, and gap junctions can be homotypic or heterotypic in construction (figure 2). In general, heteromeric gap junction assembly occurs only between connexins of the same classes. For example, Cx32 and Cx43 translated *in vitro* do not form mixed gap junction hemichannels, but when two different connexins within the same class are cotranslated, they interact to form heteromeric hemichannels (Falk *et al*. 1997, Ahmad *et al*. 1999) Within these guidelines, evidence grows that gap junctions display combinatorial connexin complexity with fascinating functional consequences (He *et al*. 1999, Cottrell and Burt 2001, Valiunas *et al*. 2000).

The topographical arrangement in the membrane of connexins 26, 32 and 43 has been established by classical biochemical and immunocytochemical approaches (Yeager and Nicholson 1996). It is most likely that all connexins are arranged with the four transmembrane segments linked by two highly conserved extracellular loops with conserved amino acid sequence identity, and a single highly variable intracellular loop (figure 3). The positioning of three cysteine residues in the two extracellular loops is inviolable (except in Cx31; Hennemann *et al*. 1992) and explains the ease with which cells of different embryonic origin form gap junctions *in vitro*, although orders of preference have been detected (White *et al*. 1995). The intracellular amino terminus is of similar length in all connexins, and the major difference between all connexins lies in the length of the intracellular carboxyl tail and the sequence motifs contained therein (table 1).

Cx43 is by far the most widely expressed connexin (table 2). A 3D electron crystallography analysis of the structure of a recombinant Cx43, containing a shortened carboxyl tail, at a resolution of 7.5 Å in the membrane plane and 21 Å in the vertical direction confirmed that the gap junction channel is dodecameric and generated by association of two hexameric

Table 1. Properties of mouse and human connexins subject to characterization. Other human connexin genomes recently identified include Cx25 (mol. mass 25, 892 D unclassified, Cx58 (mol. mass 58, 842 D unclassified) and Cx62 (mol. mass 61, 871 D, α-class). Cx26, 30 and 46 are located close together on chromosome 14; Cx40 and 50 are located on chromosome 3 (reproduced from Willecke *et al.* 2002).

Connexin	Sub-group	Chromosome	mRNA [kb]	Protein [aa]	Cyt. Loop [aa]	C-Term. [aa]	Phosph.
Cx26	β	14	2.4	226	35	18	–
Cx29		5	4.4	258	30	50	
Cx30	β	14	2.0, 2.3	261	35	55	
Cx30.3	β	4	1.8, 3.2	266	30	65	
Cx31	β	4	1.9, 2.3	270	30	65	
Cx31.1	β	4	1.6	271	30	70	
Cx32	β	X	1.6	283	35	75	+
Cx33	α	X	2.3	286	55	60	
Cx36		2	2.9	321	100	50	
Cx37	α	4	1.7	333	55	105	+
Cx40	α	3	3.5	358	55	135	+
Cx43	α	10	3.0	382	55	155	+
Cx45	γ	11	2.2	396	80	150	
Cx46	α	14	2.8	417	50	190	+
Cx47	γ	11	2.5	437	105	155	
Cx50	α	3	8.5	441	50	210	+
Cx57	α	4	3.5	505	55	275	

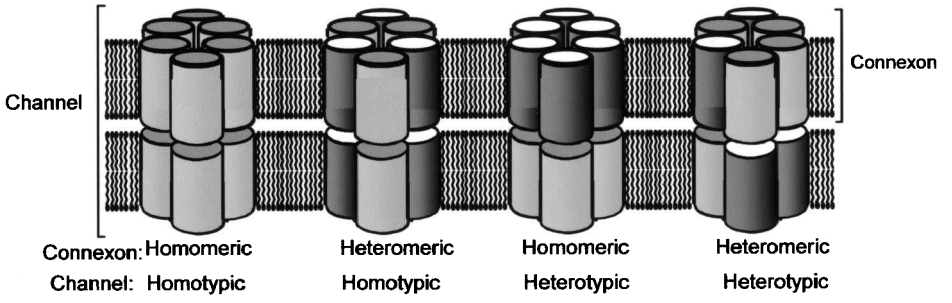

Figure 2. Possible arrangements of connexins in a gap junction channel unit.

hemi-channels. With each showing 24 rods of density in the membrane interior, it is concluded that the four transmembrane protein domains have α helical conformations (Unger *et al.* 1999). Parallel studies show that a similar overall structure is found in Cx26 gap junctions (Hand *et al.* 2002).

There is much variation in the range of connexins expressed, with morpholgically complex tissues displaying the broadest connexin profiles (table 2). For example, Cx26, 30, 30.3, 31, 31.1, 32, 37, 40, 43 and 45 are expressed in human epidermis (Di *et al.* 2001), Cx36, 37, 40, 43 and 45 in spinal cord (Kiehn and Tresch 2002) and Cx26, 32, 36, 37, 40, 43, 45 and 46 in rodent nervous system (Spray and Dermietzel 1996). Expression levels of each connexin will also vary during development. On the other hand, hepatocyte expression is confined to Cx32 and 26, eye lens to Cx46 and 50, and myocardium to Cx40, 43 and 45. Endothelium is characterized by the expression of Cx37 that is often co-expressed with Cx43 and/or Cx40.

Innexins: protein building blocks of non-vertebrate gap junctions

Surprisingly, innexins have little sequence homology to the connexins, although they are likely to display a similar topography with four predicted transmembrane domains, with intracellular amino and carboxyl termini (Phelan and Starich 2001). Innexin genes are being identified in several invertebrates, especially Drosophila bombyx, Schistocerea and Caenorhabditis elegans. At present, 24 innexins genes in C. elegans and eight in Drosophila have been identified in the respective genomes. When innexin transcripts were expressed in Xenopus embryos, gap junction type intercellular channel properties were observed (Landesman *et al.* 1999). When mRNA encoding two different innexins were co-expressed, they allowed electrical communication between cells, suggesting that hetero-oligomerization is also a feature of invertebrate gap junctions (Stebbings *et al.* 2000). Invertebrate gap junctions offer a genetic resource to more rapidly unravel the structure and function of gap junctions and the molecular nature of the intercellular signalling currency.

The functions of gap junctions

Gap junctions allow for electrical communication between cells, as demonstrated in the nervous and cardiovascular systems. In non-excitable tissues, the biochemical nature of messengers transmitted across gap junction channels under

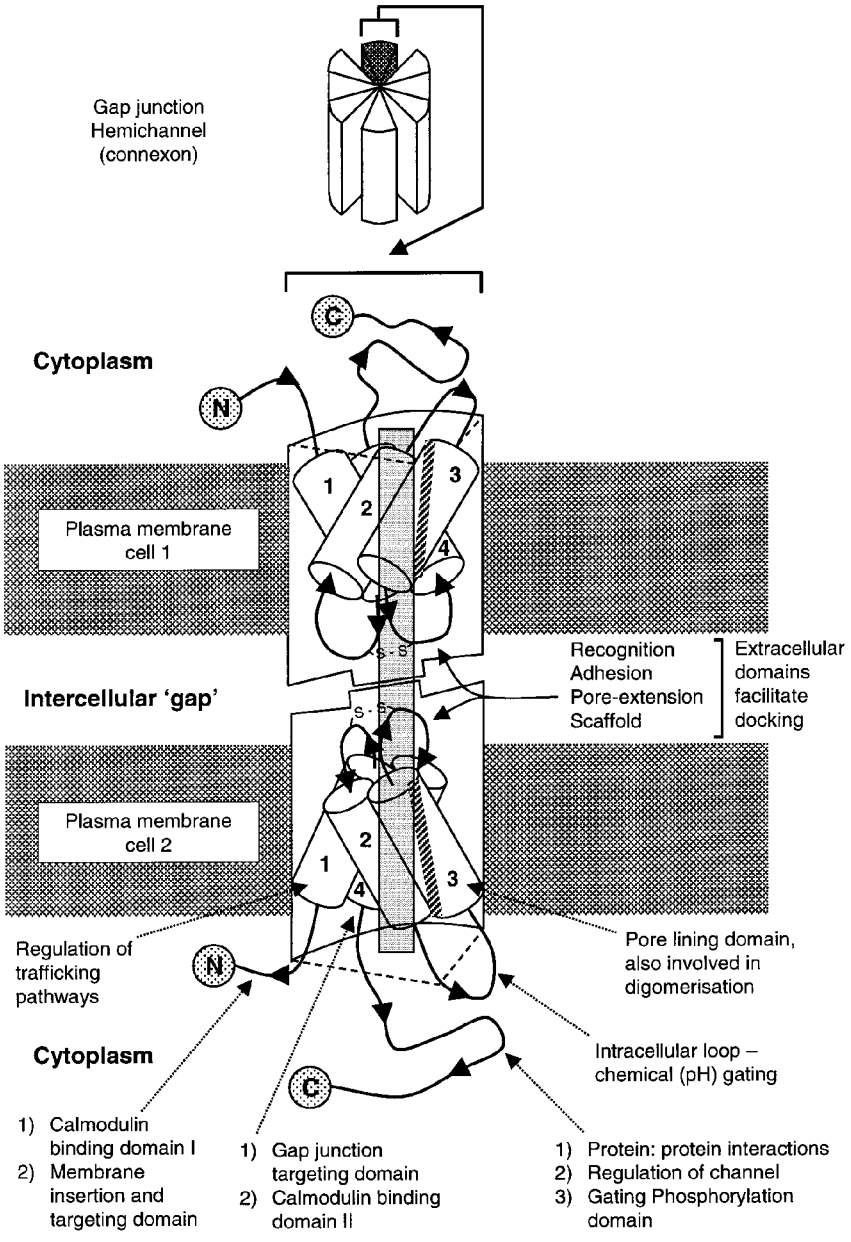

Figure 3. Drawing of two apposed connexins in a one-sixth cut out of a gap junction unit shown above. The functions of the various domains were deduced mainly from studies with Cx32 and 43. Four transmembrane domains, depicted as barrels, two extracellular loops, one intracellular loop and cytoplasmic amino and carboxyl tails are shown. The extracellular loops contain the sequences used to design the connexin mimetic peptides described in the text. Also, as discussed in the text, the precise amino acid sequences contributing to the channel wall is unclear.

defined physiological situations is not totally clear, despite an early demonstration (Lawrence *et al*. 1978) using a model system in which cardiac myocytes were coupled to ovarian granulosa cells, that cAMP diffused across gap junctions and fulfilled a clear signalling criterion.

Extensive analysis of the exchange of a range of small fluorescent dyes injected into cells has become a popular technique to establish whether cells are functionally coupled by gap junctions. These studies have uncovered charge and size discrimination of the channel within a 0.2–1.0 kD envelope, mainly in HeLa cells expressing recombinant gap junction channels constructed of various connexin isoforms (Elfgang *et al*. 1995, Cao *et al*. 1998, Nicholson *et al*. 2002). Manipulation of the connexin composition of gap junctions was shown to lead to different ionic selectivities and could result in rectification, as shown with transfected mouse cells expressing Cx26 and Cx32 (Suchyna *et al*. 1999). This diversity of connexins and their combinatorial complexity in gap junctions can result in differential channel permeability of a range of signalling molecules including cAMP (Bevans and

Table 2. Tissues expressing the various mouse connexins and the phenotypes in connexin-deficient (knock-out) mice. Human hereditary diseases associated with various connexin mutations are also indicated (assembled from White and Paul 1999, Willecke *et al*. 2002, Kelsell *et al*. 2001).

Mouse connexin	Cell and tissues with major expression levels	Phenotype(s) of Cx-deficient mice	Human hereditary disease(s)	Human connexin
	n.s.	n.s.	n.s.	hCx25
mCx26	breast, skin, cochlea, liver, placenta	lethal on ED11	sensorineural hearing loss, palmoplantar hyperkeratosis	hCx26
mCx29	myelinated cells	n.s.	n.s.	hCx30.2
mCx30	skin, brain, cochlea	hearing impairment	nonsyndromic hearing loss, hydrotic ectodermal dysplasia hair loss, nail defects and often mental deficiency	hCx30
mCx30.2	n.s.	n.s.	n.s.	hCx31.9
mCx30.3	skin	n.s.	erythrokeratoderma variabilis	hCx30.3
mCx31	skin, cochlea uterus, placenta	transient placental dysmorphogenesis	hearing impairment, erythrokeratoderma variabilis	hCx31
mCx31.1	skin	n.s.	n.s.	hCx31.1
mCx32	liver, Schwann cells, oligodendrocytes	decreased glycogen degradation, increased liver carcinogenesis	CMTX, (one of the hereditary peripheral neuropathies)	hCx32
mCx33	testis	n.s.		
mCx36	neurons retina	visual deficits	n.s.	hCx36
mCx37	endothelium, ovaries	female sterility, intensive bleeding	n.s.	hCx37
mCx39	n.s.	n.s.	n.s.	hCx40.1
mCx40	heart, endothelium	atrial arrhythmia	n.s.	hCx40
mCx43	many cell types and tissues	heart malformation and ventricular arrhythmia	visceroatrial heteroataxia?	hCx43
mCx45	heart, endothelia, neurons	lethal on ED 10.5	n.s.	hCx45
mCx46	lens	zonular nuclear cataract	congenital cataract	hCx46
mCx47	brain, spinal cord	n.s.	n.s.	hCx47
mCx50	lens	microphthalmia, zonular pulverulant and congenital cataract	zonular pulverulant cataract	hCx50
	n.s.	n.s.	n.s.	hCx59
mCx57	ovaries	n.s.	n.s.	hCx62

n.s. not studied. ED, embryonic days.

Harris 1999), NAD (Bruzzone *et al*. 2001) and inositol polyphosphates that passed about four times more efficiently through Cx32 channels than through Cx26 gap junction channels (Niessen *et al*. 2000).

Studies exploiting fluorescent calcium sensitive dyes and live-cell imaging technology have conferred on gap junctions a more direct intercellular signalling role. The demonstration that intracellular signalling, as reflected by changes in calcium levels, can be propagated rapidly and directly to neighbouring cells via gap junctions has added a new functional dimension (Sanderson *et al*. 1994, Giaume and Venance 1998). The intercellular propagation of increases in intracellular calcium levels in confluent colonies of HeLa cells expressing recombinant connexins labelled at the carboxyl terminus with the innocuous probe GFP (Green fluorescent protein) has been studied (figures 4 and 5). Intercellular propagation of calcium waves can be initiated by electrical, chemical (e.g. glutamate in astrocytes) or gentle mechanical stimuli (Isakson *et al*. 2001). Also, spot release of caged inositol phosphates provides a non-invasive technique to induce calcium wave propagation (Leybaert and Sanderson 2001). The spread of calcium released from intracellular stores in the endoplasmic reticulum to neighbouring cells was shown to be restricted to points of cell contact where gap junctions are identified by the intrinsic GFP fluorescence of the tagged connexins (figure 4, Paemeleire *et al*. 2000); the calcium wave approaches a gap junction and then appears coaxially in the neighbouring cell (figure 5(*b*)). However, it is increasingly appreciated that cells also transmit calcium signals to each other by an extracellular pathway (figure 5(*a*)) involving the release into media of ATP, possibly via open gap junction hemichannels in the plasma membrane (Cotrina *et al*. 2000, Romanello and D'Andrea 2001). Such hemichannels were first detected in cultured cells on the basis of dye uptake (Li *et al*. 1996). ATP released extracellularly triggers calcium signalling by interacting with plasma membrane purinergic receptors on neighbouring cells (figure 6), and this mechanism of intercellular spread of calcium wave propagation was exacerbated by mechanical injury (Isakson *et al*. 2001, Klepeis *et al*. 2001). Calcium has been proposed to move across the gap junctions (Saez *et al*. 1989), although it has long been appreciated that high levels of calcium close gap junctions (Rose and Loewenstein 1976). Other evidence has suggested that inositol tris phosphate (IP3) is transmitted across gap junctions (Boitano *et al*. 1992) including epithelia, where IP3 induced spread of calcium waves via gap junctions allows pacemaker cells to coordinate the overall metabolic performance of the tissue (Leite *et al*. 2002). Clearly, in the context of delineating the fine details of intercellular signalling across gap junctions in

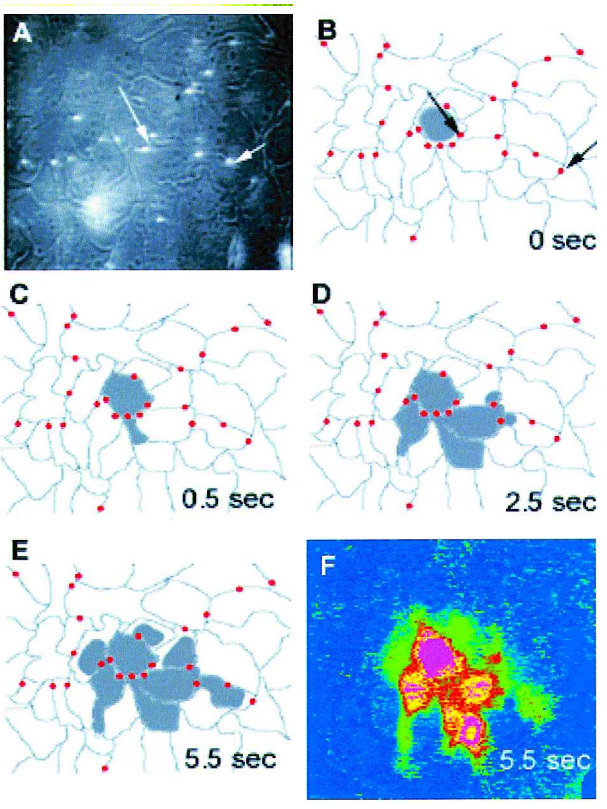

Figure 4. Digital video microscopy showing the spread of a calcium wave from the mechanically stimulated centre cell to outlined cell neighbours linked by gap junctions (white spots in (*a*), and red spots in (*b*) – (*e*) showing propagation during 5.5 s). In (*f*), the gradations in calcium deduced with fluo-3 fluorescence at 488 nm excitation are shown. In (*a*), gap junctions are fluorescent owing to expression of Cx43-GFP by the HeLa cells. Courtesy of M. J. Sanderson.

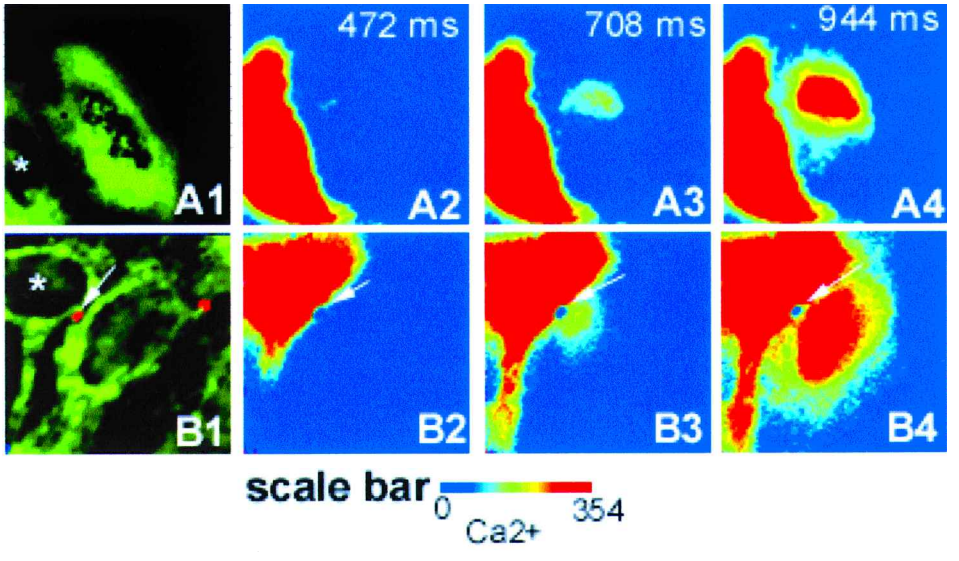

Figure 5. Calcium waves transmitted between contacting HeLa cells by two routes. In A, the spread of the calcium wave in HeLa cells lacking gap junctions occurs by release of ATP, thus involving an extracellular route. In B, the HeLa cells express fluorescent Cx43 GFP (white arrow) and the spread of the calcium wave occurs via a fluorescent gap junction (white arrow) to a neighbouring cell. This occurred in the presence of apyrase to prevent ATP acting as a mediator. A1 and B1 show the distribution of the endoplasmic reticulum using ER-tracker fluorescence (green) and (in B1) a GFP-labelled gap junction (white arrow) linking the two cells. Calcium changes are shown using fluo-3 fluorescence in pseudocolour and a scale bar. For further details, see Paemeleire *et al*. (2000). Courtesy of M. J. Sanderson.

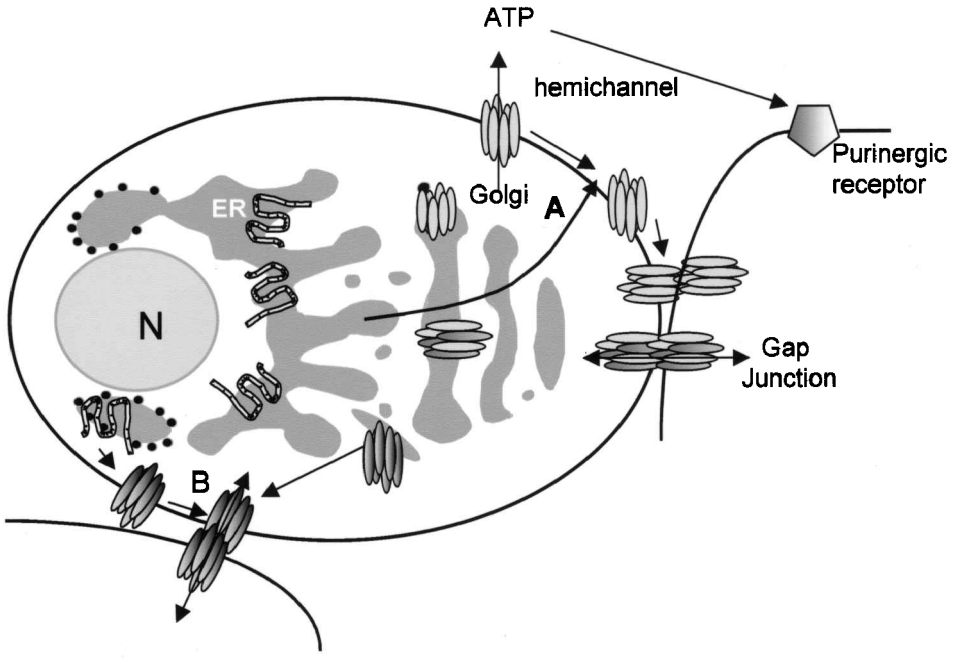

Figure 6. Multiple trafficking and assembly routes of gap junctions and dual intercellular communication pathways. In intracellular route A, Cx32 and Cx43 are co-translationally inserted into the endoplasmic reticulum, where they oligomerize into connexons and are then trafficked via the Golgi to the plasma membrane. Apposition of two connexon hemichannels results in docking to generate a gap junction. In intracellular route B, an alternative route used by Cx26 does not involve trafficking through the Golgi and possibly involves post-translational insertion into either endoplasmic reticulum or directly into plasma membranes. This complimentary route may result in oligomerization and connexon hemichannel formation after insertion into the plasma membrane. The figure illustrates how gap junctions allow direct communication and also shows connexon hemichannels across which ATP may be released, thereby providing a second connexin-dependent mechanism for the intercellular propagation of calcium waves. N, nucleus. For further details see text.

excitable and non-excitable tissues, further knowledge of the biochemical environment beneath the plasma membrane and especially the calcium levels in the vicinity of the channels (George *et al*. 1999) combined with further advances in knowledge of channel structure and gating are required.

Functional domains of connexins

Various functional properties have been assigned to specific linear domains in connexins (figure 3). Each transmembrane domain of connexins probably participates in oligomerization into hexameric connexon hemichannels. The amino acids that contribute to protein–protein interaction zones that govern their subsequent accretion after docking to generate gap junction plaques are also undetermined; indeed, other domains may also feature in the overall assembly process since many genetic mutations detected in extra membrane connexin domains impair sub-unit oligomerization (Krutovskikh and Yamasaki 2000). The oligomerization process has been analysed in cell free *in vitro* model systems and appears to proceed stepwise via dimeric and tetrameric connexin intermediates (Ahmad *et al*. 2001). Whereas homomeric oligomerization of connexins may operate as a spontaneous self-association process, the formation of heteromeric connexons of defined connexin composition is likely to be a biologically controlled process requiring the assistance of other proteins. The lateral clustering of gap junction channels in the plasma membrane is a dynamic process (Falk 2000) and many studies point to the requirement for extracellular matrix proteins in establishing cell–cell attachment and communication including cadherins (Fujimoto *et al*. 1997) and integrin $\alpha 3\beta 1$ and laminin 5 (Lampe *et al*. 1998). Whether connexons are inserted randomly into the plasma membrane or are directly inserted into the centre or edge of pre-existing clusters of gap junction plaques is unknown. Nevertheless, high-resolution fluorescence microscopy and time-lapse imaging using fluorescently tagged connexins show gap junctional plaques to be highly mobile and dynamic structures (Jordan *et al*. 2001, Falk 2000, Martin *et al*. 2001).

The third transmembrane segment has been proposed to contribute to the channel wall (Unwin 1989), and molecular models of Cx32 gap junctions appear to re-enforce this conclusion (Hulser *et al*. 1998), although evidence for a contribution of amino acid sequences from other domains has appeared (Zhou *et al*. 1997). The highly conserved amino terminal tail incorporates a putative calmodulin binding motif in Cx32 (Torok *et al*. 1997) and was shown to be necessary for the insertion of connexins into the membrane, since deleting this region as well as the first transmembrane domain prevented membrane insertion (Martin *et al*. 2000b). A voltage gating region is located at the amino terminus of Cx32 (Purnick *et al*. 2000). The two disulphide linked extracellular loops are crucial for the docking of the two hemichannels to generate a gap junctional unit, and a

number of genetic (Martin and Evans 2000, Krutovskikh and Yamasaki 2000) as well as experimental connexin mutations (Foote *et al*. 1998) show that modifications to these loops, arranged as anti-parallel β-sheets projecting into the 'gap', severely impaired docking of the connexon hemichannels. Connexin mimetic peptides designed from short specific amino acid sequences in the extracellular loops of Cx37, 40 and 43 that are likely to penetrate into this 'gap' region have been designed. These mimetic peptides operate as metabolically innocuous and reversible inhibitors of gap junctional communication and are also likely to interact with and influence the functioning of connexon hemichannels located in non-junctional areas of the plasma membrane (Warner *et al*. 1995, Evans and Boitano 2001). Structural information is relatively meagre in this outer region of the gap junction where the complementary hemichannels have docked and bridged the intercellular gap (Perkins *et al*. 1997, Unger *et al*. 1999).

Most studies on the functional anatomy of connexin domains have centred on the more accessible intracellular loop and the cytoplasmic carboxyl tail (table 1). The high variation in amino acid sequences in these regions suggests that functional differences detected between different connexins probably reside here. Deletion of most of the Cx32 carboxyl tail had little effect on its trafficking to the plasma membrane and the assembly of gap junctions (Martin *et al*. 2000b), but stepwise truncation of the tail had a graded effect on the operation of the formed gap junction channels (Castro *et al*. 1999). Similarly, most of the tail of Cx43 is not required for gap junction assembly (Ten-Broek *et al*. 2001). However, mutation of two charged amino acids located on the most proximal region of the carboxyl tail of Cx43 abolished the membrane voltage dependence of the channel (Revilla *et al*. 2000). A short region adjacent to the membrane of the Cx32 carboxyl tail incorporates a crucial gap junction targeting motif (Martin *et al*. 2000b) that also binds calmodulin (Torok *et al*. 1997). Overall, it has, so far, proven difficult to pinpoint any characteristic amino acid sorting/targeting motifs as demonstrated with several single spanning membrane proteins, and it appears likely that connexin targeting and gap junction assembly involve hierarchies of sorting signals.

The most intensively studied property of the carboxyl tail, especially of Cx43, is phosphorylation, a post-translational modification occurring at several serine, threonine or tyrosine residues. For example, phosphorylation of Ser368 on rat Cx43 *in vivo* by a protein kinase C was shown to modify single channel behaviour that correlated with a decrease in intercellular communication (Lampe *et al*. 2000) and phosphorylation of Ser364 by a cAMP-dependent protein kinase (pKa) has been identified as an important substrate (Ten-Broek *et al*. 2001). Although phosphorylation of connexins may be initiated intracellularly (Cruciani and Mikalsen 1999), phosphorylation of Cx43 is effected mainly by unidentified protein kinases when the connexons are resident at plasma membrane environs. All connexins with elongated carboxyl tails are probably phosphorylated (table 1) and, although several studies have pointed to a direct relationship between connexin phosphorylation and intercellular coupling (Kwak *et al*. 1995, Lampe and Lau 2000), the precise purpose of multisite phosphorylation of the carboxyl tail of long-tailed connexins is unclear. Recent research shows that phosphorylation is a general mechanism for setting thresholds in regulating protein–protein interactions. Progress in delineating some new functions of the carboxyl tail of Cx43 has emerged from studies in a number of laboratories designed to search for gap junction accessory proteins that interact especially with the carboxyl tail. The Cx43 carboxyl tail interacts with caveolin 1 (Schubert *et al*. 2002), the tight junction proteins ZO1 (Giepmans and Moolenaar 1998, Toyofuku *et al*. 1998) and occludin (Kojima *et al*. 1999), tubulin (Giepmans *et al*. 2001); β-catenin (Ai *et al*. 2000) and the viral protein v-src that disrupts gap junctions by phosphorylating two specific tyrosine residues, Tyr247 and Tyr 265 (Giepmans *et al*. 2001, Lin *et al*. 2001).

Cx43 tail sequences contain presumptive SH2, SH3 and PDZ protein binding domains that may facilitate many of the above interactions of Cx43 with accessory proteins, leading to the proposition that gap junctions constructed mainly of Cx43 are components of a complex nexus of proteins with extensive intracellular interactions in addition to intercellular adhesion and communication functions (Brosnan *et al*. 2001). Controversy surrounds the association of connexins with tight junction proteins. In one report, Cx26, with a short carboxyl tail of 16 amino acids, did not associate with tight junctions, whereas Cx32, with its phosphorylated 78 amino acid tail, was able to (Kojima *et al*. 2001). In contrast, Nusrat *et al*. (2000) found that a specific domain of the tight junction integral protein occludin associated with Cx26. Furthermore, gap junctions are likely to be differentially located on cell surfaces, especially in polarized epithelial cells, a process dictated by their specific connexin makeup and a consequence of different trafficking routes to the gap junction (see below).

Compared to many other membrane channels, progress in understanding the gating mechanism has been slow, possibly because of the double channel arrangement in gap junctions. Recent electrophysiological studies have aimed to simplify matters by patching gap junction hemichannels on the surfaces of single cells (Kondo *et al*. 2000, Valiunas and Weingart 2000). The gating of gap junctions is regulated by voltage, closing when a potential difference develops between the cells. Each hemichannel has a slow voltage sensitive gate (Harris 2001). The function of the voltage gate is unclear, but recent work using Cx46 that forms hemichannels in Xenopus oocytes has indicated that an activated voltage gate preferentially restricts the passage of larger fluorescent tracers, e.g. Lucifer yellow and calcein, while having little effect on the passage of smaller electrolytes (Qu and Dahl 2002). This suggests that the voltage gate allows electrical coupling while restricting movement across junctions of larger molecules with potential signalling properties. Gating of gap junctions is also regulated by intracellular acidity or calcium levels. For example, in the heart, where intracellular calcium levels can be sufficiently high to close gap junctions, this is possibly a safeguard mechanism to isolate healthy cells from dying or injured cells. Chemical regulation of Cx32, 43 and 40 has also been analysed, mainly in gap junctions forming between paired Xenopus oocytes. A ''ball and chain'' model of channel gating in which the carboxyl tail acts as a gating particle that swings around

to interact with the intracellular loop has now been proposed to operate in homologous and heterologous gap junctions (Morley *et al*. 1996, Anumonwo *et al*. 2001). In a physiological context, the effects of acute ischaemia in heart muscle leading to a local pH drop is also likely to result in channel closure and is shown to correlate with dephosphorylation of Cx43 (Beardslee *et al*. 2000). Calmodulin has also been implicated in channel regulation and shown to act either during assembly of hemichannels (Ahmad *et al*. 2001) or during channel gating (Peracchia *et al*. 2000). As discussed above, phosphorylation has been implicated in regulating channel gating in a broad range of studies, but cannot be considered as a general channel gating mechanism for gap junctions, since the smaller connexins are not phosphorylated .

Assembly and turnover of gap junctions; the involvement of multiple pathways

Membrane trafficking pathways determine the structural and biochemical composition of the membrane compartments and organelles of eukaryotic cells. Gap junctional communication and the consequent integrative responses are directly dependent on correct connexin trafficking and their assembly into functional channels. As discussed below, a number of genetically inherited communication-channelopathies are delineated and many are characterized by problems in intracellular connexin trafficking and gap junction assembly.

Most plasma membrane proteins are synthesized by membrane bound ribosomes and are delivered by vesicular trafficking from the endoplasmic reticulum via the Golgi apparatus. Connexins follow this secretory pathway but, notably, are not glycosylated during transit through the Golgi because of the lumenal topographical orientation of those amino acid motifs that are often glycosylated. The location on the secretory pathway where connexins associate into oligomeric connexon gap junction hemichannels has been controversial. A report that assembly of Cx43 into connexons was delayed until arrival in the distal regions of the Golgi (Musil and Goodenough 1993) contradicted the tenet that most membrane proteins folded and oligomerized in the endoplasmic reticulum where the necessary catalytic accessory proteins are located (Hurtley and Helenius 1989). Recent data, however, has shown that connexin oligomerization is a sequencial process commencing in the endoplasmic reticulum or its specialized domain, the endoplasmic-Golgi-intermediate compartment (ERGIC) and has been completed by arrival in the Golgi (Diez *et al*. 1999, George *et al*. 1999, Falk *et al*. 1997, Sarma *et al*. 2001).

A further intriguing aspect of gap junction biogenesis is evidence that multiple pathways exist in cells for delivery of connexins to gap junctions, especially Cx26 (Martin *et al*. 2001) (figure 6). A complementary route to gap junctions was proposed because of the inhibition of Cx26, but not of Cx32 and Cx43 trafficking to gap junctions after disassembly of the Golgi apparatus by the drug Brefeldin A (a fungal derived drug that dismantles the Golgi) or lowering the temperature to 15°C in cell cultures expressing the relevant connexins (George *et al*. 1999, Martin *et al*. 2001). Similar Golgi independence of Cx26 intracellular trafficking was observed in hepatocytes prepared from livers of Cx32 knock out mice (Kojima *et al*. 2001). However, trafficking of Cx26 to gap junctions was blocked by nocodazole, a drug that disassembles microtubules, whereas trafficking of Cx32 and Cx43 was largely unaffected (Martin *et al*. 2001). Independent subcellular fractionation approaches in guinea pig liver that separate the membrane components comprising the secretory pathway also suggested that more than one pathway existed for Cx26, since only a minor proportion of the Cx26 was delivered to plasma membranes and gap junctions via the Golgi apparatus (Diez *et al*. 1999, George *et al*. 1998). The intracellular trafficking of Cx26 may be linked to the demonstration that Cx26 was inserted into microsomal membranes in both co- and post-translational modes, as opposed to Cx32 that was inserted conventionally by a cotranslational mechanism (Zhang *et al*. 1996, Ahmad *et al*. 1999). A key role for the first transmembrane domain in determining the trafficking route and post-translational insertion into membranes of Cx26 and 32 emerged when it was shown that a single site amino acid mutation introduced into the first transmembrane domain but not the fourth transmembrane domain of Cx32 to make it resemble sequences in Cx26 more closely, conferred the post-translational membrane insertion behaviour as well as Brefeldin A insensitivity to Cx32 (Martin *et al*. 2001). Furthermore, replacement of microsomes by plasma membranes in the cell free translation system resulted in incorporation of Cx26 but not of Cx32 directly into the membranes where it oligomerized; after their transfer to liposomes, these artificial membrane vesicles showed increased permeability to small molecules, suggesting that Cx26 oligomerized to generate channels directly in plasma membranes (Ahmad and Evans 2002). The direct insertion and oligomerization of Cx26 into plasma membranes by a post-translational mechanism is reminiscent of the biogenesis of peroxisomal membrane proteins (Purdue and Lazarow 2001). Such complementary biogenetic mechanisms can allow rapid assembly of homomeric Cx26 hemichannels independently of gap junctions constructed of other connexins using the conventional secretory pathway (figure 6). A post-translational mode of insertion of Cx26 can provide a multi-trafficking basis for a number of observations such as the rapid independent synthesis of Cx26 relative to other connexins studied in the brain (Nadarajah *et al*. 1997), liver (Kojima *et al*. 1994), lactating breast (Locke *et al*. 2000), and in inflammation (Kojima *et al*. 1999, Temme *et al*. 2000). Multiple independent targeting pathways for trafficking of connexins equip cells to ensure continuous operation of intercellular communication across gap junctions, for example during cell division when the Golgi is disassembled and the secretory pathway temporarily suspended.

The diverse trafficking properties of various connexins have been analysed in the context of the rapid turnover of these proteins with half lives of 2–5 h. Many factors appear to influence the speed of connexin trafficking and turnover, including cAMP and growth factors (Paulson *et al*. 2000). G proteins also regulate Cx43 trafficking, although the directness of the implication of these information transducers acting at the under-surface of plasma membranes is unclear (Lampe *et al*. 2001).

Indeed, dynamic synthesis and turnover of connexins are an increasingly evident feature (Musil *et al*. 2000) and an indicator that gap junction number, composition and functionality are highly regulated and can be subject to rapid remodelling according to physiological requirements.

Degradation of connexins occurs in lysosomes and in proteosomes and is ubiquitin-dependent (Laing and Beyer 2000, Rutz and Hulser 2001). Cx32 has been detected in endosomes (Pol *et al*. 1997), implicating these organelles in connexin transfer from the plasma membrane to lysosomes. Morphological evidence suggests that autophagy also features in the degradation of gap junction plaques internalized in their entirety (Haftek *et al*. 1999, Jordan *et al*. 2001). Connexins interact with caveolins (proteins that associate with cholesterol, especially in lipid rafts), suggesting that more than one intracellular trafficking route may also operate in their removal from the plasma membrane. Cx32, Cx36 and Cx46, but not Cx26 and Cx50, were found to be associated with lipid rafts (Schubert *et al*. 2002). The plethora of trafficking pathways may allow for mutated connexins with aberrant folding problems to follow default pathways leading to their aggregation in intracellular degradation vesicles.

Gap junctions: roles in genetic and other diseases

Mutations in Cx32 were first shown to be associated with a peripheral neuropathy, Charcot Marie-Tooth-X (CMT-X) linked disease, a progressive atrophy of distal muscles and reduced axonal conduction by Schwann cells (Bergoffen *et al*. 1993). A number of human hereditary diseases have now been attributed to connexin mutations (table 2), and over 200 Cx32 mutations have been identified in patients with CMT-X-linked disease (Nelis *et al*. 1999), with the mutations in the DNA evenly distributed throughout the various connexin domains. Some of these Cx32 mutations appear to affect the function of gap junctions or hemichannels that provide short-cut cytoplasmic pathways in the adaxonal and perinuclear Schwann cell cytoplasm in the myelin sheath; these radial pathways have been calculated to be a million times shorter than a circumferential route (Balice-Gordon *et al*. 1998). Mutations in Cx26, of which over 30 have been identified, account for about half of the inherited non-syndromic deafness in the western world and are emerging as the first practical genetic marker of inherited hearing loss (Steel and Kras 2001). The most common mutation is a recessive mutation (35delG) that results in premature stopping of protein translation; a further six dominant mutations are found in the first extracellular loop of Cx26 (Rabionet *et al*. 2000). Cx26 and Cx31 appear to play a critical role in the physiology of hearing by controlling the circulation via gap junctions of ions to the stria vascularis, where potassium is pumped back to the cochlear endolymph to restore a high potassium level. Mutations in Cx26 are also increasingly associated with skin diseases (Kelsell *et al*. 2001, Rouan *et al*. 2001). In the lens, mutations in Cx46 and Cx50 are linked with cataract abnormalities (Pal *et al*. 2000). In contrast, few mutations have been detected in Cx43, despite intensive investigations to search for a mutational basis to proposed relationships between this connexin and growth control and malignancy. Exceptionally, mutations are reported on phosphorylated serines on the carboxyl tail of Cx43 and claimed to be associated with viscero-atrial heterotaxia, a severe heart malformation and a generalized failure to establish left/right symmetry as well as in hypoplastic left heart syndrome (Dasgupta *et al*. 2001). Also, an amino terminal mutation on Cx43 has been shown to be associated with non-syndromic deafness (Liu *et al*. 2001). Mutations in Cx37 are also rare, although some were detected in hepatic angiosarcomas induced by vinyl chloride, but these are probably single nucleotide polymorphisms (Kumar *et al*. 2000).

Cx32 mutations detected in neuropathies and Cx26 mutations associated with hearing defects are widely distributed throughout the relevant genes, making it difficult to associate them specifically with the pathology and/or its severity. To study the molecular basis of the pathology, connexin mutations have been selected for detailed biochemical analysis in model systems, especially in cultured mammalian cells that express very low levels of connexins and are unable to exchange dyes and in Xenopus oocyte models. One objective in such studies has been to determine the effects of selected mutations on the assembly and functional properties of gap junctions. Three major classes of mutations are recognized. Class 1 includes those mutations that have no discernible effect on the normal operation of the gap junction channel, as detected by electrical properties of the junctions or by dye coupling. Class 2 mutations result in altered gating properties of gap junctions. Class 3 mutations are those studied mainly in model mammalian cell systems that result in the failure of connexins to traffic and assemble into gap junctions with the unassembled connexins accumulating mainly in the endoplasmic reticulum. The last class has formed the basis of a number of studies using either selected naturally occurring genetic mutations or those that are scientist-induced. Approaches adopted include studies of whether the mutated connexins are correctly integrated and inserted with the correct topography into membranes, either in cell free *in vitro* translation systems or when expressed in non-communicating cultured cells, and whether functional gap junctions are generated as measured by transfer of fluorescent dyes between confluent cells or by electrophysiological techniques. These approaches have provided new information on how mechanisms of assembly of gap junctions are modified in these communication-opathies (Deschenes *et al*. 1997, Oh *et al*. 1997, Omori *et al*. 1996, Martin *et al*. 1999, 2000a, van Slyke *et al*. 2001). As found in other well-studied channelopathies associated with various diseases, especially the delta 508 mutation of the Cystic Fibrosis Receptor Channel (CFTR) protein, unassembled connexins that accumulate in the endoplasmic reticulum are rapidly degraded, ultimately by proteosomal mechanisms, possibly involving transfer into large intracellular vesicles. Intriguingly, there appears to be a link between the expression of the CFTR protein and connexins, possibly related to emerging general roles for connexins in mediating inflammatory responses (Chanson *et al*. 2001, Oviedo-Orta *et al*. 2001).

Misregulation of connexin expression is a widespread feature in cell coupling changes associated with various diseases. Extensive studies have been pursued of relation-

ships between cell communication across gap junctions and cell growth and division. Inspired by a predictive review (Loewenstein 1979), many studies (reviewed by Trosko and Ruch 1998, Klaunig and Ruch 1990; Omori *et al*. 2001) have searched for links between connexin expression levels and gap junction modifications in cell growth and cancer, especially in cells expressing Cx26 and Cx43. For example, low expression of Cx43 has been postulated as an independent marker for breast cancer tumours (Laird *et al*. 1999). Although a large number of studies have been based on measurements of expression levels of connexin mRNA or protein as possible indicators of oncogenesis, they have often provided contradictory results in different systems. Recently, the intracellular position of connexins and their levels relative to those at gap junctions has been highlighted as another of the factors influencing cell growth (Krutovskikh *et al*. 2000). However, intracellular 'hidden' connexins are also emerging as a feature of epidermal stem cells, and could be a characteristic feature of their pluripotential properties in which a developmental trigger is required to initiate intracellular trafficking and assembly of connexins into functional gap junctions and, thus, facilitate heterocellular interactions (Matic *et al*. 2002). The availability of tissues from mice in which connexin expression has been suppressed by homologous recombination techniques has allowed possible relationships between gap junctions and high proliferation rates to be analysed. Although it has not been possible so far to study mice with limited expression of Cx26, owing to their death *in utero*, it appeared at first that Cx32 deficient mice were not abnormal; for example, liver regeneration was unaffected. However, it has since been shown in Cx32 deficient mice that glucose mobilization from glycogen was reduced after stimulation of sympathetic nerves (Nelles *et al*. 1996) and susceptibility to liver carcinogens was increased, although loss of Cx32 expression did not prime hepatic tumour development (Evert *et al*. 2002, Willecke *et al*. 2002).

Several studies indicate that Cx43 expression by astroglial cells that play important neuroprotective roles, is modified in neurodegenerative brain pathologies. For example, in Altzeimer's disease, an increase in βA4 amyloid plaques corresponded to higher Cx43 or Cx30 immunoreactivity (Nagy *et al*. 1999). In Huntington's disease, characterized by neuronal death in basal ganglia, the distribution of Cx26 and Cx32 was unchanged, but Cx43 levels increased especially in astrocytes, and the distribution of this connexin became coincident with plaques (Vis *et al*. 1998). In a laboratory model of Parkinson's disease, Cx43 expression increased in the striatum in parallel with glial fibrillar protein staining (Rufer *et al*. 1996). Although the results suggest that cell communication across glial cell gap junctions is one of the critical parameters modified by these pathologies, this is not surprising in view of the fundamental importance of gap junctional communication in effecting syncytial-type behaviour of glial cells. Nevertheless, these advances may lead to new approaches to treat cerebral ischaemia and stroke, by manipulating after injury the gating of gap junctions and possibly permeability of connexon hemichannels in astrocytes (Lin *et al*. 1998, Kirchhoff *et al*. 2001).

In the heart and cardiovascular system, Cx40, 43 and 45 are located at gap junctions that underpin intercellular current flow and ensure synchronous contraction of myocytes (Saffitz 2000). Conduction defects cause fatal ventricular arrhythmias and connexin abnormalities are present in diseased hearts. Changes in Cx40 protein expressed at high levels in the atrium are causally associated with an increased risk of developing atrial fibrillation after coronary bypass surgery (Dupont *et al*. 2001).

Connexin defects in transgenic mice

Genetic ablation of connexins has provided new and sometimes surprising insights into the roles of various connexins and is contributing towards the setting up of a code to decipher the functions of specific connexins. Ablation of Cx43 results in heart malformations, especially the obstruction of the right ventricular outflow tract (Reaume *et al*. 1995), retardation of the migration of cardiac neural crest cells (Lo *et al*. 1999) and a susceptibility to ventricular arrhythmia. However, a tissue-specific cardiac deletion of Cx43 yields a structurally normal heart, but one that is prone to a lethal ventricular arrhythmia associated with slow myocardial conduction (Gutstein *et al*. 2001). Deletion of Cx40 leads to atrial arrhythmias although results obtained by different research groups indicate a complex integrative physiology (Kirchhoff *et al*. 2000, Bevilacqua *et al*. 2000; Simon *et al*. 1998; Lerner *et al*. 2000). Targeted deletion of both Cx43 and Cx40 result in a variety of heart malformations, especially defects in the atrio-ventricular junction, with developmental faults also detected such as growth of the right ventricle and septation (Kirchhoff *et al*. 2000). Deletion of Cx45 resulted in mice that died at ED 10.5 with an altered cardiac cushion (Kumar *et al*. 2000) and generally defective vascular development especially resulting from thinner blood vessels (Nishii *et al*. 2001). In summary, mouse knock-out models confirm that gap junctions play crucial roles in heart morphogenesis and cardiac conduction, but determining clear roles in cardiac development and function is hampered by the fact that cells express multiple connexin isoforms (Lo 2000; Lo *et al*. 1999, Willecke *et al*. 2002).

In the vasculature, gap junctions present in endothelial and smooth muscle cells provide a rapid mechanism of communication that can allow synchronized changes in diameter of small and large arteries (Sandow and Hill 2000, Berman *et al*. 2002). Endothelial-smooth muscle signalling is complex, and the molecular nature, although unresolved, features heterocellular calcium signalling across gap junctions (Dora 2001, Chaytor *et al*. 2001). An endothelial specific deletion of Cx43 resulted in hypotension secondary to elevated NO and bradycardia (Liao *et al*. 2001), further emphasizing the importance of gap junctions in endothelial control of vascular tone.

Further tissue-specific knockouts and double knockouts are needed to clarify the functional roles of specific connexins. Thus far, many of these approaches support the concept of tissue-specific compensation occurring between multiple connexins (Plum *et al*. 2001), especially, for example, the apparent interdependence of Cx43 and Cx40 in cardiac morphogenensis (Kirchhoff *et al*. 2000).

However, this does not always follow, as studies using double knockouts in various tissues are showing connexin independence. For example, independent functions of connexins is illustrated by the apparent lack of co-ordination of Cx32 and Cx26 expression and gap junction assembly in livers (Kojima *et al*. 2001), where there are homomeric and heteromeric connexons present (Diez *et al*. 1999). Also, studies of mice with double knockouts and targeted replacement by 'knockin' approaches show, for example in lens, that Cx50 is required for cell growth whereas Cx46 provided non-specific restoration of intercellular communication (White 2002). The likelihood of the presence of homomeric and heterotypic channels in most tissues complicate analyses of genetically induced functional deficits and genetic corrections, and illustrate why efforts are needed to better understand the molecular architecture of gap junction intercellular communication channels.

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