BEYOND THE GAP: FUNCTIONS OF UNPAIRED CONNEXON CHANNELS

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Gap junctions consist of intercellular channels that connect the cytoplasm of adjacent cells directly and allow the exchange of small molecules. These channels are unique in that they span two plasma membranes — the more orthodox ion or ligand-gated channels span only one. Each cell contributes half of the intercellular channel, and each half is known as a connexon or hemichannel. Recent studies indicate that connexons are also active in single plasma membranes and that they might be essential in intercellular signalling beyond their incorporation into gap junctions. **[AU: edit of preface OK?]**

METAZOA
A subkingdom of animals including all multicellular organisms with differentiated tissues.

HEMICHORDATES
A phylum of bilaterally symmetric marine animals with gill-slits to the pharynx.

ACTION POTENTIAL
A self-propagating opening of voltage-gated channel along the length of a cell.

GRANULOSA CELL
A cell type found in mammalian ovarian follicles that surrounds the developing oocyte.

NON-SYNDROMIC DEAFNESS
Deafness that is unaccompanied by other clinical manifestations.

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doi:10.1038/nrm1072

Intercellular channels, which cluster together to form [**AU:OK?**] gap junctions, are phylogenetically ancient and are found to join virtually all METAZOAN cells, from cnideria and nematodes to HEMICHORDATES and mammals. This long evolutionary history has allowed their adaptation to many varied uses, which range from diffusion of morphogens in *Hydra*[1] to the adaptation to dark in the retina[2].

This diversity of function is reflected in the diversity of connexins. Six connexin molecules form each hexagonal connexon hemichannel, and, in conventional gap junctions, two connexons from adjacent membranes join — leaving a gap of ~2–3 nm — to form each intercellular channel (BOX 1).

There are over 20 connexins, each of which confers different properties of conductance and regulation on the channels they form[3–5]. For example, connexin-dependent differences in channel permeability have been shown for various ions and dyes[6–8] and for signalling molecules such as cyclic AMP and cyclic GMP[9]. Furthermore, most cells express several connexins, and the mixing of connexins has been shown to create new channel properties that are not evident in any of the parent channels[10–12].

Several studies, though, have detected connexons at the cell surface that are not part of an intercellular gap junction[13–16] and there is evidence that connexons function 'beyond the gap'. To provide a context for understanding these extra-junctional connexons, it is important to illustrate briefly the diversity of functions of intercellular channels.

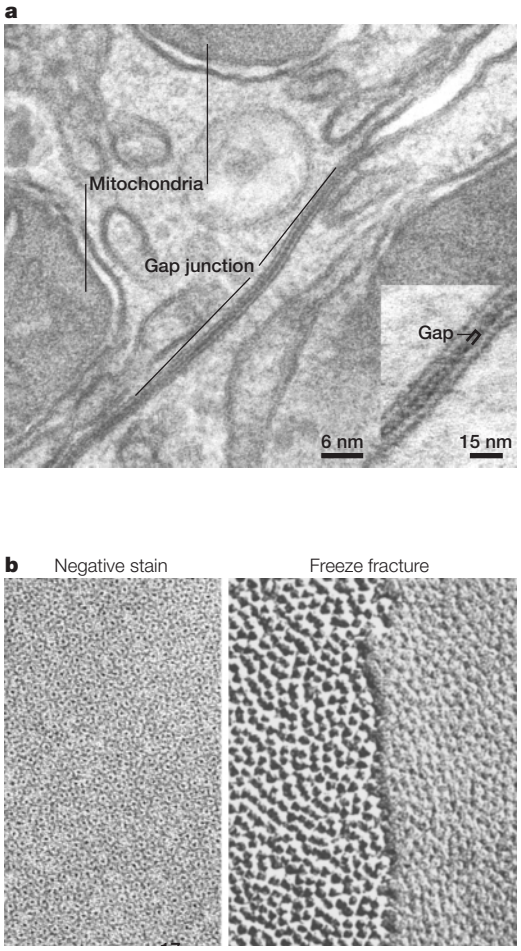
Historically speaking, intercellular channels allow the cell–cell transfer of ACTION POTENTIALS between excitable cells, which was the basis for their discovery[17]. New functional information has emerged from studies of human connexin mutations and knockout mice. In general, the knockout studies have shown that gap-junctional intercellular channels function to coordinate cellular activity. Examples include the intercellular propagation of second messengers[18], bidirectional signalling between oocytes and GRANULOSA CELLS to coordinate maturation of the ovarian follicle[19], and the maintenance of osmotic balance in lens homeostasis[20,21]. The study of human mutations has shown that defects in connexin-based intercellular channels underlie several genetic diseases, although the mechanisms are not yet understood. These include a peripheral neuropathy known as X-linked Charcot–Marie–Tooth disease that results from demyelination[22] (BOX 2); skin disorders[23]; congenital cataracts[24–27]; and more than half of all cases of NON-SYNDROMIC DEAFNESS[28]. Detailed reviews of human connexin diseases and knockout mice have been published recently[29–32].

An important point to bear in mind, though, is that attributing specific cellular functions to connexons is hampered by the lack of specific inhibitors for either connexons or intercellular channels. In addition, the

Box 1 | Connexins, connexons and gap junctions

Connexins are four-pass integral membrane proteins, with both the amino and carboxyl termini facing the cytoplasm. They are generally referred to by their predicted molecular weight[117] (for example, connexin32 (Cx32) is predicted to be ~32 kDa) but they also have a Greek designation according to the order of their discovery[118] (for example, Cx32 is also known as β_1). These proteins oligomerize intracellularly into hexamers (which are known as connexons) that are then inserted into the plasma membrane[15]. By mechanisms that are unclear, the connexons pair with their counterparts in an adjacent cell, and form the axial intercellular channel. The intercellular channels cluster in maculae that are known as gap junctions (figure, part **a** [**AU:OK?**]). The small projections of the extracellular domains of the paired connexons results in a 2–3 nm separation (gap) between the junctional membranes[119] that is visible by electron microscopy (figure, part **a**). Freeze fracture and negative staining provide images of the intercellular channels in gap junctions (figure, part **b**). A 7.5 Å three-dimensional model of the intercellular channel has been published using low-dose Fourier microscopy[120].

The intercellular channels allow the cell–cell diffusion of ions and small molecules, which thereby creates an ionic and metabolic syncytium in the compartments of interacting cells. The ionic conductance allows rapid intercellular spread of action potentials in excitable cells, such as heart cells and neurons. In non-excitable cells, many different small molecules and second messengers might diffuse from cell to cell. It is likely that cyclic AMP passes through gap-junctional intercellular channels that join ovarian granulosa cells with oocytes, which maintains meiotic arrest[121]. In general, however, although cAMP and ATP have been shown to pass through gap-junctional channels that join cells in culture[122,123], it is not yet known which small molecules functionally permeate gap junctions in whole animals.



expression of exogenous connexins in cultured cells might be accompanied by the upregulation of other proteins, which further confounds [**AU: hinders?**] interpretation. These caveats notwithstanding, the presence of connexons in the non-junctional membranes of cells is now well established and offers the opportunity to study their potential physiological role.

Evidence for extra-junctional connexons

Evidence for extra-junctional connexons comes from biochemistry and electrophysiology. SURFACE-LABELLING, SUCROSE GRADIENT FRACTIONATION and CROSSLINKING studies have shown the presence in the plasma membrane of connexons that are incorporated into an intercellular channel or a junctional plaque[14,15]. Similarly, antibodies that are directed against extracellular connexin epitopes can block the assembly of gap junctions in living cells[33]. As the extracellular space in gap junctions is too narrow for an antibody to gain access[34], these studies confirm that connexons are present outside the junctional plaque before they assemble into gap junctions. Although it was known previously from *in vitro* studies that active connexons could be reconstituted into artificial lipid bilayers[35], until recently it was assumed that extra-junctional connexons were in a closed state, as their opening could result in membrane depolarization and the depletion of small molecules from the cytoplasm.

But are they active? Evidence for active channels composed of extra-junctional connexons was first obtained by the *in vitro* expression of cloned connexins. Expression of connexin46 (Cx46) in *Xenopus laevis* oocytes resulted in membrane depolarization, which was followed by eventual cell death[13]. The oocytes also became permeable to Lucifer yellow, a low-molecular mass fluorescent compound that is used widely to assess junctional communication. Together, these observations indicate the presence of open extra-junctional [**AU:OK?**] connexons. Depolarization activated Cx46-containing connexons, as did decreases in extracellular Ca^{2+} (REF. 36), and so it seems that connexon activity is modulated in a manner that is similar to other, more orthodox, ion channels.

Studies of retinal neurons also provided evidence for open, extra-junctional connexons. Similar to the oocytes that expressed Cx46, cultured TELEOST HORIZONTAL CELLS developed large voltage-sensitive plasma-membrane

SURFACE-LABELLING
The chemical modification of cell-surface molecules using reagents that are membrane impermeant.

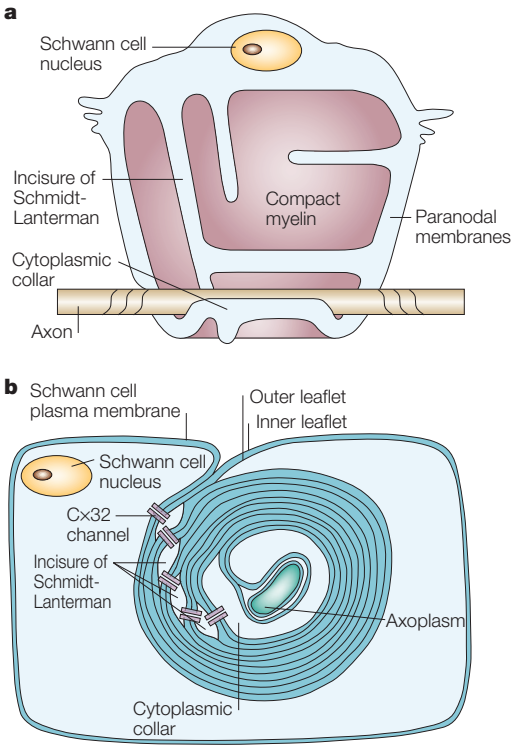
SUCROSE GRADIENT FRACTIONATION
The biochemical separation of subcellular components on the basis of their buoyant densities.

CROSSLINKING
The use of bifunctional reactive compounds to covalently couple molecules that are in close proximity.

TELEOST HORIZONTAL CELL
A cell type that is found in fish retinas.

Box 2 | 'Reflexive' gap junctions and Charcot–Marie–Tooth disease

X-linked Charcot–Marie–Tooth disease is a demyelinating disorder of the peripheral nervous system that is characterized by the slow progressive loss of both motor and sensory functions, particularly in the distal extremities. It is caused by mutations in connexin32 (Cx32), which is expressed abundantly in SCHWANN CELLS. More than 240 different mutations in Cx32 have been associated with this familial disorder[124]. Curiously, myelinating Schwann cells do not form gap junctions with other cells but concentrate Cx32 in PARANODAL MEMBRANES and the INCISURES OF SCHMIDT-LANTERMAN[22] (see figure, part a, which shows an 'unrolled' myelin sheath). The paranodes and incisures are regions where myelin is not compacted and cytoplasm connecting the cytoplasmic collar region with the Schwann cell body is retained (figure, part a). These tubular cytoplasmic connections are extremely long and small in diameter, so diffusion would be an inefficient mechanism for exchanging molecules between the cell body and the periaxonal cytoplasm. It is believed that the cell solves this complex anatomical problem by establishing 'reflexive' gap junctions between the turns of the cytoplasm-filled tubes (see figure, part b, which shows a 'rolled up' myelinating Schwann cell). This idea was tested by simultaneous injection of junction-permeant and junction-impermeant dyes into the Schwann cell body. Permeant dyes accumulated rapidly in the periaxonal region, whereas impermeant dyes failed to move[125]. So in the peripheral nervous system, intercellular channels couple different parts of the same cell, rather than different cells, and loss of these channels results in the eventual failure of myelin homeostasis. Reproduced with permission from REF. 32 © Annual Reviews (1999).



SCHWANN CELL
A glial cell in the peripheral nervous system that is responsible for the myelination of axons.

PARANODAL MEMBRANE
A plasma membrane that surrounds non-compact areas of Schwann cells that are found immediately adjacent to nodes of Ranvier.

INCISURE OF SCHMIDT-LANTERMAN
A spiraling region of non-compact myelin that joins the periaxonal with the perinuclear cytoplasm in myelinating glia.

NOVIKOFF HEPATOMA CELLS
A cell line derived from a liver tumor that is used in the study of gap-junctional intercellular communication.

ASTROCYTES
One of several classes of glia (non-neuronal support cells) that are found in the central nervous system.

VENTRICULAR MYOCYTE
A cell that is found in the ventricles of the heart.

N2A NEUROBLASTOMA CELLS
A cell line derived from a brain tumour that has very low endogenous connexin expression, and is useful for studying the [AU:OK?] introduction of exogenous connexins.

OLEAMIDE
(9(Z)-octadecenamide). A sleep-inducing lipid that was first isolated from the cerebrospinal fluid of sleep-deprived cats. It rapidly and reversibly closes gap-junctional channels.

GLYCYRRHETINIC ACID
A lipophilic saponin isolated from licorice roots that blocks gap junction channels and connexons. [**AU: should this be α-glycyrrhetinic or 18 α-glycyrrhetinic acid?**]

conductances and became permeable to Lucifer yellow when extracellular Ca^{2+} was lowered[16]. Strikingly, the non-junctional conductances were sensitive to changes in the same conditions that affected intercellular channels between horizontal cells — notably, changes in voltage, cAMP, cGMP, dopamine and intracellular pH[37]. Together, these data indicate that the extra-junctional connexons were probably responsible for the horizontal-cell membrane conductances. Subsequently, connexons that function as transmembrane channels have been observed in NOVIKOFF HEPATOMA CELLS[38], ASTROCYTES[39] and VENTRICULAR MYOCYTES[40]. Further members of the connexin family have also been shown to induce conductance through single membranes in *Xenopus* oocytes and in mammalian cell-expression systems — connexin50 (Cx50; REF. 41), connexin45 (Cx45; REF. 42), skate connexin35 (Cx35; REF. 43) and *Xenopus* connexin38 (Cx38; REFS 44,45). So the formation and activation of connexons seems to be a general property of connexins in various cell types.

Clearly, uncontrolled connexon activity could have deleterious consequences. Indeed, overexpression of Cx46 in oocytes causes lysis, presumably owing to the uptake of water through open connexons by osmosis[13]. This unusual behaviour of Cx46 does not normally occur in mammalian cells, and is only seen in frog oocytes because of [AU:OK?] differences between the extracellular Ca^{2+} concentrations in amphibians. Raising the extracellular Ca^{2+} concentration to that of mammals resulted in connexons made of Cx46 remaining closed, which prevented oocyte lysis[36]. This Ca^{2+}-dependent connexon behaviour is consistent with the behaviour of [AU:OK?] open connexons in teleosts that was outlined above[16]. Lowering the concentration of extracellular Ca^{2+} has been shown to open water channels in the membranes of N2A NEUROBLASTOMA CELLS that have been transfected with Cx43, and results in a change in cell volume[46]. The Ca^{2+}-dependent volume change was shown to require connexin expression and to be inhibited by the gap-junctional intercellular-channel blockers OLEAMIDE and α-GLYCYRRHETINIC ACID [**AU: glossary term to include α? Should this be 18 α?**], which indicates that these water channels are composed of open connexons. As discussed, the mechanism of chemical blockade of intercellular channels is not understood, and is probably indirect. It follows, therefore, that the use of chemicals to block connexons is similarly correlative.

Responding to experimental manipulation. Connexons show similar responses to experimental manipulation as do intercellular channels. The well-known sensitivity of many intercellular channels to cytoplasmic acidification, which was originally discovered in *Xenopus* embryos[47], is also displayed by connexons that are composed of Cx46 (REF. 48).

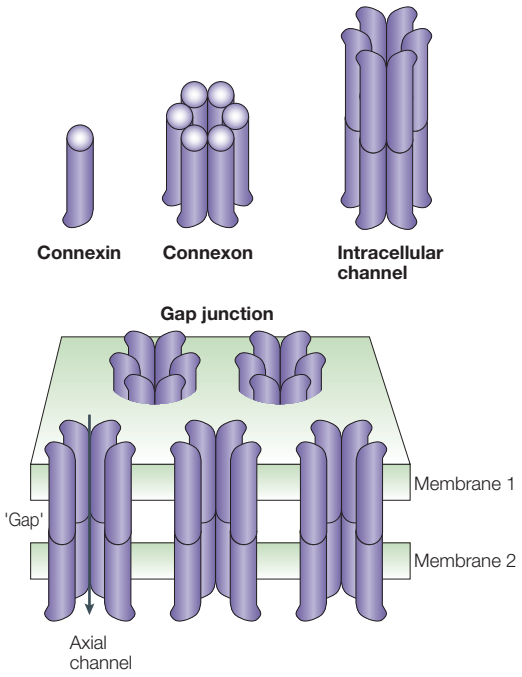


Figure 1 | **Connexins, connexons, intercellular channels and gap junctions.** A diagram showing the relationships between the connexin monomer, the hexameric assembly of connexins into a connexon and the intercellular joining of two connexons to form a dodecameric intercellular channel. Clusters of intercellular channels are known as gap junctions owing to the minute extracellular 'gap' that separates the apposed plasma membranes. Each intercellular channel provides an axial channel (arrow) that interconnects the cytoplasm of the apposed cells directly.

Comparative studies with Cx46 in *Xenopus* (and its chicken counterpart, Cx56) showed that the pH sensitivity, ion selectivities and permeability properties of the intercellular channel can be explained by modelling two connexons joined head-to-head, as they are [AU:OK?] in an intercellular channel (FIG. 1), indicating that the connexon structure does not markedly change when it is incorporated into an intercellular channel[49,50]. In another example, the extra-junctional connexons detected at the surfaces of teleost horizontal cells show sensitivities — to voltage, cAMP, cGMP, dopamine and intracellular pH[37] — that are similar to the intercellular channels, again indicating that the connexon structure does not change grossly on incorporation into an intercellular channel. Detailed data comparing the biophysical properties of the connexon with those of the intercellular channel are not available for other members of the connexin family.

Blocking the channels. The experimental use of intercellular-channel blockers to close connexons has been popular. In a comprehensive study of connexons composed of Cx46 and Cx50 in *Xenopus* oocytes, it was shown that selected anion-channel blockers — for example, flufenamic acid (FFA) and 5-nitro-2(3-phenyl-propylamino)benzoic acid — inhibited connexons that were composed of eitherconnexin [AU:OK?] with a high affinity[51]. Interestingly, although octanol, niflumic acid (NFA) and diphenylamine-2-carboxylate closed connexons composed of Cx50, these agents were unable to close Cx46 connexons when used at the same concentrations. FFA and NFA have been shown to block the current of endogenous *Xenopus* connexons[52] that are known to be composed of Cx38 (REF. 45).

Taken together, several conclusions can be drawn from the published data. First, chemical blockers of intercellular channels might be active on connexons, although the mechanisms of action are not known and in some cases might be indirect. Second, as the mechanisms of action of these agents are not known, they might close or open other membrane channels (or influence unknown intracellular targets), which rules out any direct correlation of channel or connexon closure with changes in cellular behaviour. Finally, there could be differences in the sensitivity of connexons that are composed of different connexins to individual reagents, which would require each member of the connexin family to be specifically tested.

Closed or open? In general, although connexons are probably on the surfaces of most cells, they remain primarily in a closed state. Connexins have been transfected into many cell lines, but there has been no consequent evidence of connexon activity[53–57]. Curiously, connexon activation might be a common response to metabolic inhibition[58–60]. For example, ischaemia activates connexons in myocardial cells, a response that would, at least in theory, promote arrhythmias and further myocardial injury, which would exacerbate the problem. But how could the activity of large, non-selective channels in the plasma membrane contribute to the normal functioning of a cell?

Intercellular propagation of Ca^{2+} waves

In many cell types, various stimuli increase the concentration of intracellular calcium ($[Ca^{2+}]_i$), which can propagate as a 'wave' into surrounding cells. Previously, this propagation was assumed to be due entirely to gap-junctional intercellular channels. Certainly, wave propagation has been well-correlated with connexin expression. For example, C6 GLIOMA CELLS, which have low endogenous connexin levels, show an increase in the $[Ca^{2+}]_i$ in response to mechanical stimulation, but they do not propagate waves unless they are transfected with Cx43 (REFS 61,62). Conversely, pharmacological inhibitors of gap-junction channels block wave propagation among astrocytes[63,64] and other cell types[65,66]. These and other studies imply that propagation of Ca^{2+} waves requires connexins. As inositol 1,4,5-trisphosphate ($InsP_3$) triggers the release of Ca^{2+} from the endoplasmic reticulum (ER) into the cytoplasm, this propagation is dependent on the diffusion of $InsP_3$ through the intercellular channels of gap junctions[67].

C6 GLIOMA CELLS
A cell line derived from a glioma that is used to study intercellular communication and Ca^{2+} waves.

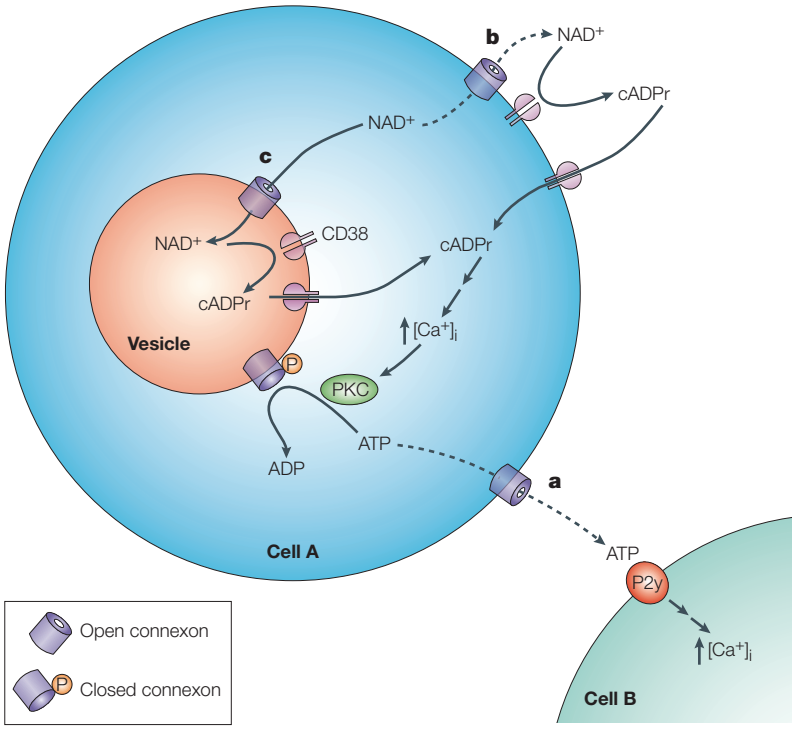


Figure 2 | **Connexons and nucleotide transport. a** | Activation of connexons in the astrocyte plasma membrane causes the release of ATP into the extracellular space where it activates P2y purinergic receptors on adjacent cells to initiate a Ca^{2+} wave. **b** | Active connexons allow cytosolic oxidized nicotinamide-adenine dinucleotide (NAD^+) to gain access to the ectoenzyme CD38, which converts it to cyclic ADP-ribose (cADPr). The movement of extracellular cADPr into the cytosol might be affected by a transport activity of CD38, although other mechanisms are possible. **c** | Connexons could also allow transport of cytosolic NAD^+ into the lumen of intracellular vesicles, where it is exposed to CD38 and converted to cADPr. On CD38-mediated translocation to the cytosol, cADPr stimulates an increase in intracellular Ca^{2+} ($[Ca^{2+}]$). This rise in Ca^{2+} could facilitate the phosphorylation of connexin by protein kinase C (PKC), resulting in closure of the connexon and limiting the access of NAD^+ to the active site of CD38. This schematic model is a potential mechanism of connexon function.

Connexons and ATP release

Recent studies provide clear evidence for an alternative mechanism for connexin-dependent Ca^{2+} wave propagation in astrocytes[68,69] and OSTEOCYTES[70] that might involve extra-junctional connexons. In this model, wave propagation relies on the release of ATP into extracellular space. ATP binds P2 PURINERGIC RECEPTORS on an adjacent cell, which stimulates the production of $InsP_3$. In turn, this raises the $[Ca^{2+}]_i$, which activates more ATP-releasing channels, thereby propagating a wave. In support of this idea, it has been shown that Ca^{2+} waves are accompanied by the release of ATP, and wave propagation is blocked by purinergic receptor antagonists[66,68,71–75]. In another study, stimulating the release [**AU: production?**] of $InsP_3$ in single cells resulted in an intercellular Ca^{2+} wave. In addition to blocking this wave with α-glycyrrhetinic acid [**AU: 18 α?**], the wave was also blocked by Gap 26, a peptide that corresponds to a connexin extracellular loop. This peptide was inactive in blocking gap-junctional intercellular channels, implying that the peptide only had access to the extracellular portions of the connexins in a non-junctional connexon[76].

If this alternative Ca^{2+} wave propagation does not require intercellular channels, why does there seem to be a requirement for connexins? One explanation is that regulated connexons provide a mechanism for ATP release (FIG. 2a). Several different lines of evidence support this idea. First, levels of connexin expression correlate well with the magnitude of ATP release in transfected cell lines[61] and cultured astrocytes[77]. Second, astrocytes accumulate low-molecular-mass membrane-impermeant dyes in their cytoplasm, which is consistent with the presence of active connexons[39]. Third, it has been shown that both the propagation of Ca^{2+} waves and the release of ATP from astrocytes are potentiated by extracellular application of quinine (which activates some connexons[43]) and are repressed by the gap-junction blocker FFA[78]. In the latter study, the reduction of extracellular $[Ca^{2+}]$ both activated whole-cell currents and increased markedly the intracellular uptake of a junction-permeant dye. However, ATP release was not blocked by the inhibitors of intercellular communication, 18 α-glycyrrhetinic acid [**AU: just α?**] and octanol[61], which would argue against an involvement of connexons. However, these studies used transfected cell lines, whereas those using the gap junction blocker used primary cultures of astrocytes, which might provide some explanation for the discrepancy. Similarly, oleamide has been shown to block intercellular channels but not to interfere with transmission of the calcium wave in rat glia[79]. So connexon activity might have a role in ATP release and Ca^{2+}-wave propagation in astrocytes, but without specific gap-junctional channel blockers, the data remain only correlative.

Connexons and NAD^+ release

Connexons might be involved in the release of signalling molecules other than ATP. Evidence for paracrine signalling through connexon-mediated release of nucleotides comes from studies of cyclic ADP-ribose (cADPr), which is a second messenger that induces Ca^{2+} release from the ER[80]. cADPr is thought to modulate the activity of Ca^{2+} channels of the ER separately from the $InsP_3$-regulated Ca^{2+} channels that were discussed above. cADPr is synthesized from the oxidized nicotinamide-adenine dinucleotide (NAD^+) by the ectoenzyme CD38, the active site of which is extracellular. CD38 might also function as a cADPr-specific transporter that allows extracellular cADPr to reach the cytosol. However, until recently, the mechanism by which CD38 gains access to NAD^+ was a puzzle. Zocchi *et al.*[81] described a passive, temperature-independent NAD^+ transport system in the plasma membrane of 3T3 fibroblast cells which could be blocked by 18 α-glycyrrhetinic acid [**AU: just α?**] or by treatment with antisense-Cx43 oligonucleotides[82]. Furthermore, the transport activity co-isolated with Cx43 and was specifically inhibited by an anti-Cx43 antibody when reconstituted into LIPOSOMES. These data indicate that active connexons (FIG. 2b) might provide the mechanism for

OSTEOCYTE
A differentiated bone cell that is trapped in lamellar bone. Osteocytes are interconnected by gap junctions by processes that extend through long canaliculi.

P2 PURINERGIC RECEPTOR
A cell-surface ATP receptor.

LIPOSOME
A unilamellar vesicle.

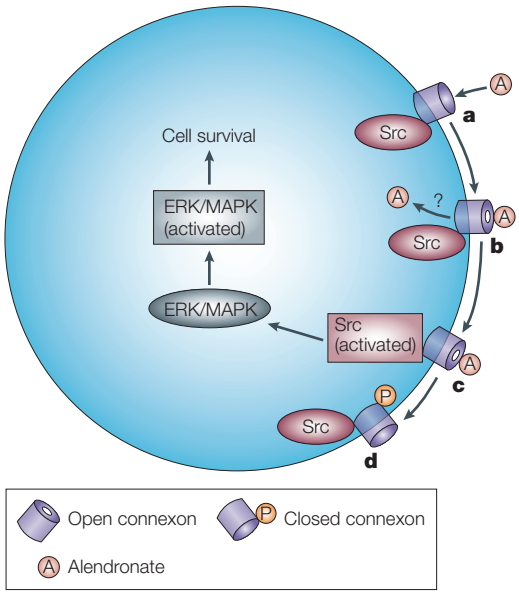


Figure 3 | **Connexon-mediated apoptosis. a** | Exposure to alendronate activates plasma-membrane connexons containing Cx43. **b** | It is possible that alendronate binding to Cx43 results in connexon opening. It is probable, but has not been shown, that alendronate traverses the open connexons to enter the cytosol. **c** | Subsequently, Src is activated, which in turn activates extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) to facilitate cell survival. How Src is activated is not yet clear. However, Cx43 constitutively binds Src and the conformational change induced by connexon opening could activate it. **d** | Conveniently, feedback inhibition of this pathway would result from Src phosphorylation of Cx43 that inhibits gap-junction channel activity. This schematic model is a potential mechanism of connexon function. **[AU: is the connexon in part a phosphorylated? If not then should we include it in the key as an inactive/closed connexon?]**

NAD^+ release, which could constitute a general mechanism for calcium-related signal transduction between cells[83,84].

It has also been proposed that a link between Cx43 and CD38 constitutes a mechanism for regulation of $[Ca^{2+}]_i$[85]. CD38 is contained in intracellular vesicles during exocytic transport to the plasma membrane and after ligand-stimulated endocytosis[86]. After their synthesis, Cx43 connexons are also transported by an exocytic pathway[14] and could therefore be present in the same post-Golgi vesicles as CD38. Such vesicles could generate cytoplasmic cADPr using the vesicle lumen as the equivalent of the extracellular space (FIG. 2c). When cytosolic NAD^+ reaches the lumen of the vesicle through an open connexon, it would be converted to cADPr by the ectodomain of CD38. The cADPr would be transported to the cytoplasm by either a CD38-dependent transport activity (as discussed above) or an uncharacterized transporter, which would result in increases in cytosolic Ca^{2+}. Bruzzone *et al.*[85] provide indirect evidence that feedback regulation of this pathway results from activation of protein kinase C and the subsequent phosphorylation of Cx43, which closes the connexon channels (FIG. 2). So, the enzymatic activity of the vesicle and its ability to generate cADPr would be regulated by the activity of the connexon channel.

Connexons and osteocyte survival

Bone is an organ that is characterized by rapid turnover — this requires constant cell birth and death. Bone cells are connected by gap junctions and express both Cx43 and Cx45 (REFS 87–89). OSTEOBLAST dysfunction in *cx43*-knockout mice[90] leads to a delay in bone mineralization and skeletal defects[91], which indicates a potential role for connexins in bone growth and remodelling. Recent studies of bisphosphonates, a class of drugs that is used in the treatment of bone diseases[92], implicates connexon activity in OSTEOGENESIS. Bisphosphonates inhibit apoptosis of osteoblasts and osteocytes[93], but not OSTEOCLASTS[94]. For example, in an experimental model of bone-cell death, apoptosis induced in cultured bone cells by dexamethasone can be blocked by the bisphosphonate alendronate[95]. The mechanism underlying this activity requires activation of extracellular signal-regulated kinases/mitogen-activated protein kinases (ERKs/MAPKs) and Src. It is not clear how alendronate affects the activity of the kinases but evidence indicates that connexons are involved. First, the anti-apoptotic effect of alendronate can be inhibited by 18 α-glycyrrhetinic acid [**AU: just α?**], carbenoxolone, or oleamide —agents that all [**AU:OK?**] block connexon channels[96]. Second, the anti-apoptotic activity is observed in cells that have been plated at a very low density, so gap-junctional intercellular channels cannot be involved[96]. Third, inhibition of apoptosis is seen in cells that express Cx43, but not in those that are derived from the *cx43*-knockout mouse, which indicates that there is a requirement for connexin expression. In addition, the ability to inhibit apoptosis can be restored to $cx43^{-/-}$ cells by transfection with Cx43 (REF. 96). Fourth, exposure to alendronate induces dye [**AU: just a marker dye?**] uptake from the medium, which indicates that alendronate activates connexons[97].

Connexons and kinases. Connexon activity can be linked to the activation of kinase cascades. It is known that the carboxy-terminal cytoplasmic domain of Cx43 interacts directly with, and is phosphorylated by, Src, and that this results in the inhibition of gap-junctional intercellular channels[98]. So, a probable mechanism is that the connexons are opened transiently by alendronate (FIG. 3a), which causes a conformational change in Cx43 possibly leading to Src activation by unknown mechanisms and the initiation of a signal cascade (FIG. 3c). Cell survival would be favoured by both activation of ERK/MAPK, possibly owing to regulation of anti-apoptotic Bcl-2 (REFS 99,100), and the rapid closure of connexons by Src-mediated phosphorylation of Cx43 (FIG. 3d). It is of note that connexons composed of other connexins cannot restore alendronate sensitivity. As mutations of Cx43 that eliminate Src binding do not restore sensitivity, this probably reflects the unique ability of Cx43 among

OSTEOBLAST
A bone cell that synthesizes and secretes osteoid, which is the extracellular matrix of bone. Osteoblasts differentiate into osteocytes.

OSTEOGENESIS
The process of bone formation.

OSTEOCLAST
A mesenchymal cell that can differentiate into a bone-degrading cell.

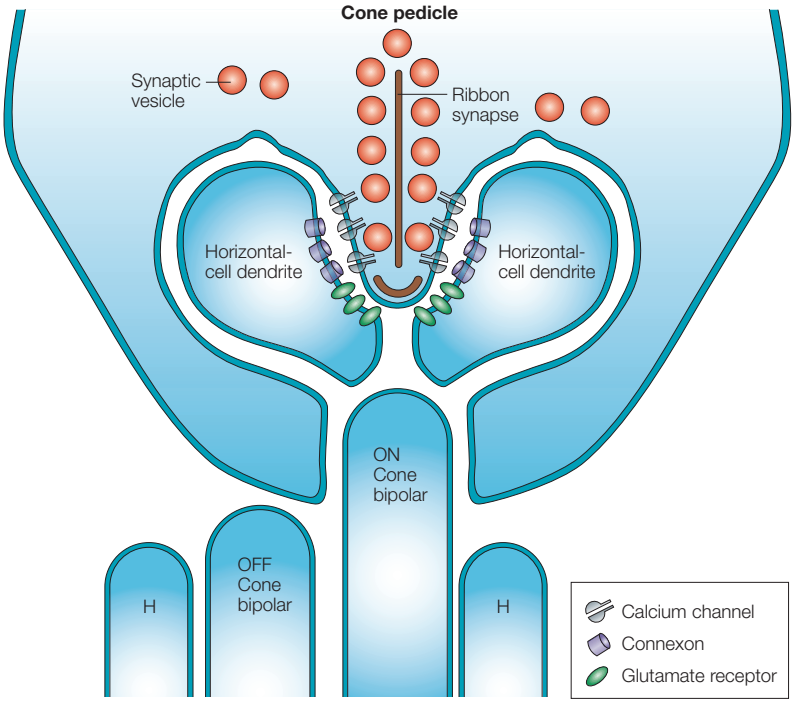


Figure 4 | **A diagram of a cone pedicle with an invaginating synapse.** Depolarization of the horizontal-cell dendrites that are deeply embedded in the cone pedicle, which is characterized by its unique ribbon synapse and associated synaptic vesicles, might open connexons in the horizontal-cell membrane. This would result in the flow of current into the horizontal cell and the creation of a negative extracellular potential in the confined region of the invaginating synapse. This local potential change would cause activation of voltage-sensitive Ca^{2+} channels in the pedicle membrane (through depolarization), increasing the release of glutamate. Processes from an ON cone bipolar and OFF cone bipolar **[AU: Are these the same as the ON-centre and OFF-centre ganglion cells mentioned in the text?]** also synapse with the cone pedicle, each of which might also interact with horizontal (H)-cell processes (interactions are not shown; see REF. 126 for more details). Modified with permission from REF. 126 © Elsevier Science (1998).

connexins to bind Src. It is not known if alendronate binds directly to the extracellular domains of Cx43 and induces connexon opening, or if an indirect signalling pathway is involved. It remains to be determined if alendronate actually uses the connexon to gain access to the cytoplasm (FIG. 3b) — this is feasible, as its molecular mass is only 250 Da.

Presumably, connexons have not evolved as receptors for alendronate, and so this advantageous cellular response indicates that there are endogenous signalling molecules that, similar to alendronate, could stimulate an anti-apoptotic activity that is important in the coordination and regulation of bone turnover. As embryonic fibroblasts that express Cx43 can also be protected from dexamethasone-induced apoptosis by bisphosphonates, the use of connexons in protection from programmed cell death might extend to other cell types as well. Potential links of apoptosis with connexon activity indicate that the relationship between tumour suppression, cancer chemotherapy and connexons should be re-examined. For example, the expression of Cx43 in glioblastoma cells not only suppresses transformation and tumorigenicity, but also increases sensitivity to chemotherapeutic agents, such as etoposide and Taxol[101].

PEDICLE
The ending of a cone cell that interacts with other neurons in the retina.

Connexons and neuronal signalling

Extra-junctional connexons could participate in the neuronal signalling that underlies centre-surround antagonism in the retina. Individual retinal ganglion cells respond to illumination in particular regions of the retina, which are known as the receptive field. In most cases, this field consists of two parts; a central zone and a surrounding annulus. Illumination of the central zone with a small spot of light will excite an ON-centre ganglion cell, whereas illumination of the surrounding annulus will inhibit it[102–104]. Surround antagonism is an important mechanism for sharpening ganglion-cell resolution and responsiveness to contrast. This antagonism is mediated by the interaction of horizontal cells with cone photoreceptors[104–106]. A given horizontal cell might interact with photoreceptors in both the centre and the surround because their processes extend over large lateral distances. Surround antagonism results from cone input to horizontal cells in the centre that drive horizontal-cell negative feedback to cones in the surround and inhibit their responsiveness to light stimuli.

Although the involvement of the horizontal cell in cone surround responses is well established, the cellular mechanisms are disputed. It has generally been accepted that the release of the neurotransmitter γ-aminobutyric acid (GABA) from horizontal cells underlies negative feedback to cones. However, a confounding issue is the absence of conventional presynaptic vesicles in the horizontal cell to mediate GABA release. A plausible release mechanism that involves the GABA transporter has been proposed[107] but has not been conclusively shown.

An alternative model, which is based on ephaptic feedback (neural transmission at non-synaptic contact sites) has been proposed[108]. Because horizontal-cell dendrites are deeply embedded in the cone PEDICLE, with resultant tortuous, narrow intercellular spaces, the resistance of the extracellular space is relatively high. So glutamate receptors on the horizontal-cell dendrites could form a sink for the current that would modulate the extrasynaptic potential, which in turn could modulate cone voltage-activated Ca^{2+} currents that are responsible for the release of transmitter. Kamermans *et al.*[109] showed that glutamate receptors do function as current sinks, but under physiological conditions their effect on photoreceptor signalling would be positive, not negative. They proposed a variation of the model, in which active connexons are the basis of the current sink (FIG. 4). Opening of connexons in horizontal-cell dendrites in the cone pedicle would result in a negative extracellular potential owing to the flow of current through the connexons. As discussed above, this would in effect depolarize the pedicle, opening Ca^{2+} channels and increasing glutamate release, the same effect as reducing light stimulus to the cone.

The data that support this idea are provocative. First, in teleosts, Cx26 is found exclusively on the dendrites of horizontal cells and, furthermore, is strategically located adjacent to the sites of glutamate

release in the cone pedicles[110]. Because cones and horizontal cells neither establish gap junctions nor display electrical coupling with each other, Cx26 is probably present on the horizontal-cell membrane in connexons. Second, as discussed above, dissociated teleost horizontal cells have active connexons in low Ca^{2+} medium[16]. Third, using carbenoxolone, a non-specific blocker of gap-junctional channels, (assuming that this agent will also block connexons, as reviewed above) Kamermans *et al.*[109] were able to reversibly block the feedback-induced inward Ca^{2+} currents in cones while still maintaining their light response. Carbenoxolone also hyperpolarized the horizontal cells and reduced their light responses. The positive shift of the Ca^{2+} current indicates that the connexon inactivation, and not the hyperpolarization of the horizontal cell, is responsible for the block of feedback responses to cones.

Conclusion and future directions

The presence of connexons in plasma membranes has now been established in many cellular systems. Connexons are the precursors of gap-junctional intercellular channels[14] and have been shown to accumulate by lateral membrane diffusion at the periphery of gap junction plaques[57,111]. The first evidence of open connexons[13,16] has been followed by studies that implicate them in several cellular activities, including Ca^{2+} wave propagation, ERK/MAPK signalling in anti-apoptotic protection and ephaptic neuronal communication.

A common weakness of the studies reviewed here is their dependence on pharmacological agents to block gap-junctional channels. Unfortunately, none of these agents has been shown to interact specifically with connexins, connexons or intercellular channels and it is almost certain that the mechanisms of their actions are indirect. In many cases, other signalling pathways are known to be affected. On the assumption that different agents operate by different mechanisms, some studies use a panel of blockers in parallel experiments to confirm the findings. Nevertheless, it is clear that further exploration of connexon function will require broader approaches. A second weakness of many studies that are cited here is that altered connexin expression is known to result in the upregulation of many other cellular genes and in cytoskeletal changes[61,112], which also confounds functional interpretations.

Specific antagonists would provide key reagents for the study of connexon function. In one report, antisera raised against the extracellular Cx43 structure resulted in connexon closure[39]. Synthetic peptides that correspond to the extracellular sequences of connexins have been used in studies designed to interfere with the assembly of intercellular channels[33,76,113–115], but, with the exception of one study[76], the effects of these reagents on connexon function have not been explored. This approach might provide insights that are useful in the development of specific pharmacological agents. Alternatively, chemical libraries could be effectively screened using automated fluorescence assays that are designed to detect compounds that can block connexon-permeable dye uptake in isolated cells[116]. The combination of specific channel blockers with gene ablation approaches would provide the most compelling evidence for connexon activity.

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Acknowledgements.
[AU: Any acknowledgements? For your information, it's not journal style to include grant numbers, but it is OK to acknowledge funding bodies]

Online links

DATABASES
The following terms in this article are linked online to:
OMIM: http://www.ncbi.nlm.nih.gov/Omim/
X-linked Charcot-Marie-Tooth disease
Swiss-Prot: http://www.expasy.ch/
Bcl-2 | CD38 | Cx26 | Cx32 | Cx35 | Cx38 | Cx43 | Cx45 | Cx46 | Cx50 | Cx56 | Src

FURTHER INFORMATION
Author's laboratory: [AU: would you like to include a link to your research homepage?]
Access to this interactive links box is free online.

Daniel A. Goodenough received his Ph.D. in Anatomy in 1970, and David L. Paul recevied his Ph.D. in Cell and Developmental Biology in 1983, from Harvard Medical School, USA. D.A.G. and D.L.P. began their research collaboration in 1974 at the Department of Anatomy, Harvard Medical School. These investigators have studied the cell biology of gap-junction-mediated intercellular communication, initially focusing on the biochemical characterization of isolated gap junctions from liver and lens. Since the isolation of connexin cDNA and the initial characterization of the connexin family, they have studied the phenotypes resulting from targeted ablation of mouse connexin genes. At present, D.A.G. is Takeda Professor of Cell Biology, and D.L.P. is Professor of Neurobiology, at the Harvard Medical School. [**AU: edit OK?**]

- Gap junctions are aggregates of intercellular channels that join adjacent cells. Intercellular channels are composed of a pair of connexons, each of which is a hexamer of connexin proteins. As precursors to the intercellular channels, connexons can be found in the plasma membranes of cells. In some cellular systems, however, connexons might function independently of gap junctions.
- Although there are data implicating gap-junctional intercellular channels in the cell–cell transmission of Ca^{2+} waves, it is also clear that these waves can spread by paracrine signalling owing to the extracellular release of ATP that binds to purinergic receptors. Evidence is accumulating that the connexon might provide a regulated channel that is responsible for the ATP release.
- The bisphosphonate alendronate has been shown to have an anti-apoptotic effect on bone cells. Recent data indicate that connexons might be the receptor for alendronate, transducing a survival signal through the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathway.
- A long-standing puzzle has been the interaction of retinal horizontal cells with cones as part of centre-surround antagonism. The interaction between these cells might involve connexons. The voltage-induced opening of connexons in the horizontal-cell membrane might function to create a negative extracellular potential in the invaginating synapses of the cone pedicles, opening Ca^{2+} channels and stimulating glutamate release. [**AU: edit of summary OK?**]

Links

OMIM

X-linked Charcot-Marie-Tooth disease

http://www.ncbi.nlm.nih.gov/htbin-post/Omim/getmim?search=charcot+marie+tooth+x-linked&field=title

Swiss-Prot

Bcl-2
http://ca.expasy.org/cgi-bin/niceprot.pl?P10415
CD38
http://ca.expasy.org/cgi-bin/niceprot.pl?P28907
Cx26
http://ca.expasy.org/cgi-bin/niceprot.pl?P29033
Cx32
http://ca.expasy.org/cgi-bin/niceprot.pl?P08034
Cx35
http://ca.expasy.org/cgi-bin/niceprot.pl?Q92107
Cx38
http://ca.expasy.org/cgi-bin/niceprot.pl?P16864
Cx43
http://ca.expasy.org/cgi-bin/niceprot.pl?P17302
Cx45
http://ca.expasy.org/cgi-bin/niceprot.pl?P36383
Cx46
http://ca.expasy.org/cgi-bin/niceprot.pl?Q9Y6H8
Cx50
http://ca.expasy.org/cgi-bin/niceprot.pl?P48165
Cx56
http://ca.expasy.org/cgi-bin/niceprot.pl?P29415
Src
http://ca.expasy.org/cgi-bin/niceprot.pl?P12931