Gap-Junction Proteins in Retinal Development: New Roles for the “Nexus”

Gap-junction channels, the cytoplasmic proteins that associate with them, and the transcriptional networks that regulate them are increasingly being viewed as critical communications hubs for cell signaling in health and disease. As a result, the term “nexus,” which was the original structural name for these focal intercellular links, is coming back into use with new proteomic and transcriptomic meanings. The retina is better understood than any other part of the vertebrate central nervous system in respect of its developmental patterning, its diverse neuronal types and circuits, and the emergence of its definitive structure-function correlations. Thus, studies of the junctional and nonjunctional nexus roles of gap-junction proteins in coordinating retinal development should throw useful light on cell signaling in other developing nervous tissues.

The gap junction was first identified by electron microscopy as a quasi-crystalline array of membrane-spanning particles, the “nexus” (28), which could couple adjoining cells into electrical and metabolic networks (3). Later studies showed that each particle in such an array consists of two hemichannels with aqueous pores (3). Later studies showed that each particle in such an array consists of two hemichannels with aqueous pores joined end-to-end, one traversing the plasma membrane of each of the participating cells (FIGURE 1). Each hemichannel is a cylindrical hexamer of transmembrane proteins encoded by genes from one of two distinct families: a large and diverse family of connexins (39), found only in chordates (75), and a non-homologous, more ancient and even more diverse family composed of the innexins, found only in invertebrates, and the pannexins (74), whose importance for vertebrate gap-junctional communication is still controversial (90, 95).

The cells of an embryo have many ways of communicating. They can do so, for example, through diffusable ligands such as parts of or secreted proteins that act on membrane-bound receptors (12, 15), through membrane-anchored ligands such as ephrins, which may also have additional “reverse-signaling” receptor activity (47), or through soluble gases such as nitric oxide to which cell membranes present no barrier (1, 82). Historically, as each of these signaling modes has been investigated in turn, it has diluted the apparent importance of the modes discovered previously. Indeed, so many of these intercellular signals coexist and interact in the continuously changing embryo that it is challenging to design experiments that can tease out a specific role for each one.

It has been particularly hard to assign specific developmental roles to gap junctions. They transmit ions and small molecules of many different classes, including universal second messengers such as ionic calcium, inositol 1,4,5-triphosphate, and the cyclic nucleotides and intracellular peptides, at least up to nonamer size (71). At least one unexpectedly massive (17 kDa) but highly elongated signaling protein, calmodulin, has been reported to pass through them (19), and evidence has recently been presented in support of the idea that they may also transmit information-dense oligonucleotides such as siRNAs (112, 119), which could, in principle, allow them to coordinate gene expression directly across multicellular populations. But the limited molecular selectivity that makes these channels so versatile also makes it hard to discover which aspect of the cross-border traffic is critical at any moment and what message it is carrying. Gap-junction permeability has also been troublesome to control experimentally, because no truly selective pharmacological agents have yet been found. Those most commonly used appear to act indirectly, through the lipid microenvironment, and affect other transmembrane channels to varying degrees as well (42). Thus, although gap junctions were known to have important developmental roles long before the details of other signaling systems emerged, a better understanding of those roles has had to await more precise investigative tools.

In recent years, the combined use of electrophysiology, live-cell imaging, and molecular biology has brought new insights. Gap junctions are now known to influence the proliferation of neuronal progenitor cells and the migration of postmitotic neuroblast (32) and to coordinate patterned physiological activity before the formation of chemical synapses (123). (For a time-frame for these events, see FIGURE 2). Channels constructed from different members of the connexin family have selective and distinctive docking and gating properties, potentially allowing communication to be regulated independently within restricted tissue.
compartments (21, 116). Undocked hemichannels in extra-junctional regions of the cell membrane can also have functional signaling roles (35). Connexons also have actions besides transmembrane conductance, mediated in some cases by the homophilic adhesive properties that allow hemichannels on adjacent cells to dock in pairs (33). In other cases, intracellular interactions with other cytoplasmic proteins at junctional membrane microdomains form a type of protein complex that has also been described as a nexus (31). Most recently, statistical analysis of large gene arrays has shown that perturbing the expression of a single connexin gene (Gja1, coding for connexin Cx43) during development can have a measurable and predictable impact on the expression of hundreds of other genes from many functional classes, linked together into a complex transcriptional network with important implications for development, physiology, and pathology (99). At least one connexin gene may act as a central focus or hub for such a network, taking the nexus concept to a third, transcriptomic, level of meaning that complements the existing anatomical and proteomic levels.

The retina is an outstanding model for central nervous system (CNS) development, being derived from the neural tube like the rest of the CNS but having the distinct experimental advantages that it is more accessible, more amenable to study in vitro, and structurally simpler than most CNS regions. It is also highly laminated, to the extent that it has been called "nature’s brain slice" (113). Moreover, its structure-function correlations, circuits, synapses, computational mechanisms, and neuronal types are known in more detail than those of any other brain region. Studies of gap-junctional roles in retinal development, therefore, ought to throw useful light on cell signaling in other developing nervous tissues. In the sections that follow, we consider their contribution so far, and their potential.

**Gap Junctions in the Optic Cup**

The first retinal neurons are generated in the wall of a two-layered epithelial structure called the optic cup, formed by the infolding of a balloon-like outgrowth from the neural tube. As long ago as 1972, when intercellular communication across the optic cup was being considered a candidate experimental system to specify the central targets of individual retinal ganglion-cell axons, gap junctions were observed by electron microscopy in and between the cells of the neural retina (the inner epithelial layer) and the pigment epithelium (the outer layer) (29, 30). The prevalence of large gap junctions among proliferating cells in the neural retina was recognized soon afterwards (38, 44). Extensions of these studies from Xenopus frogs to chicks and then monkeys confirmed that these gap-junctional arrays are largest and most numerous in the period when ganglion cells are being born and that they connect the neuroepithelial precursors of these cells not only to their neighbors in the inner layer of the cup but also across the plane of the newly obliterated ventricular space to the pigment epithelium of the outer layer (37, 43, 94, 110). In these three species, spanning the limbed vertebrates, gap junctions in the proliferative zone were found to become smaller and fewer as differentiation progressed and the pool of cycling precursors was depleted. There have been few functional studies of intercellular coupling at these early developmental stages. Lucifer Yellow injections into the large cells of a tetraploid strain of Xenopus frogs revealed dye coupling between neuroepithelial neighbors up to stage 30 but not between neurite-bearing ganglion cells at later stages (87). A later study in the chick combined intracellular injections with immunohistochemistry to compare the distribution of tracer coupling with that of connexins Cx26, Cx32, and Cx43 (5). However, the injections were made into differentiating ganglion cells, identified by their axons, so they could reveal only patterns of coupling that included such cells. Many of the coupled cells, especially at later stages, had the shapes and locations of differentiating inner retinal neurons. However, some of the cells that were filled indirectly while neuronal production and migration were still occurring remained coupled after neuronal progenitor cells or migrating postmitotic neurons, suggesting that gap-junctional coupling might link newborn neurons to their older siblings. Expression of these three connexins did not appear to correlate with the extent of tracer coupling; this is no longer surprising because, although Cx43 dominates communication within the neuroepithelial layer and pigment epithelium in all species studied to date, it is now known that Cx45 dominates in both chicks (5) and Xenopus embryos. Data from some experimental and some control studies, in which the role of Cx45 in the proliferating retina is being explored, will be considered.

**Roles in Central Nervous System Development**

The first indication that connexins might regulate cell proliferation came from studies of retinal ganglion-cell development in embryos derived from a small, spontaneously tetraploid strain of Xenopus (Xenopus laevis). Xenopus laevis is the amphibian from which the experimental retinal ganglion-cell system was developed. When control and sense-oligonucleotide-injected embryos were compared, it was found that ganglion cells were decreasing in number in sense-treated retinas with fewer and smaller, more distributed, more anisotropic gap junctions than in control retinas. That experiment suggested that gap junctions were required to coordinate cellular coupling at these early developmental stages. That experiment, and other studies, was the first indication that gap junctions might be involved in coordinating neural interactions between the retinal ganglion cells. The experiments were criticized because they did not consider the possibility that the effects observed were experimental artifacts, produced by the injection of small RNA molecules into the living embryos. Experiments in which chicken embryos were injected with connexin mRNA into stage 10 embryos of the same species showed that the injection of connexin Cx43 mRNA into the brain cell mass of chicken embryos did not affect embryonic or postnatal development, and that the mRNA was not elaborated after injection. In the chick, injection of mRNA for the small connexins Cx26 and Cx32 (which are not expressed in chicken ganglion cells), into the brain cell mass did not cause any detectable uncoupling, and neither did injection of control sense mRNA, although the mRNA injected contained an inactivating mutation that prevented translation into connexin protein. The conclusion was reached that the small connexins Cx26 and Cx32, which are not expressed in chicken ganglion cells, are not involved in specifying the functional development of the retina. In general, the conclusions reached from these experiments are supported by results obtained since then, and the general conclusion is that gap junctions are not involved in regulating cell proliferation in the retina. However, a role cannot be entirely ruled out because the experimental protocol used to study the connexin mRNA injections involves premature gastrulation in which the cells are not truly specific to retinal development.

The experiments described in this review suggest that gap junctions are functionally involved in regulating the proliferation of retinal ganglion cells in Xenopus. The significance of this role is that such interactions might be required to coordinate the proliferation and differentiation of retinal ganglion cells, and that the role might be conserved in all vertebrates. The results also suggest that the role of gap junctions in the retina is not to promote but to inhibit neuronal cell death, because the injection of connexin mRNA into the brain cell mass of the chick did not affect neuronal survival. The results also suggest that the role of gap junctions in the retina is not to promote but to inhibit neuronal cell death, because the injection of connexin mRNA into the brain cell mass of the chick did not affect neuronal survival. The results also suggest that the role of gap junctions in the retina is not to promote but to inhibit neuronal cell death, because the injection of connexin mRNA into the brain cell mass of the chick did not affect neuronal survival. The results also suggest that the role of gap junctions in the retina is not to promote but to inhibit neuronal cell death, because the injection of connexin mRNA into the brain cell mass of the chick did not affect neuronal survival.
now known that other connexins such as Cx36 and Cx45 dominate in the differentiating inner retinae of both chicks (54) and mice (55). More recent studies, in which the roles of gap-junctional coupling among proliferating retinal progenitors have been explored experimentally, are discussed in following sections.

**Roles in Controlling Neuroepithelial Proliferation**

The first indication that gap-junction proteins might regulate the proliferation of retinal neuronal progenitors came from a study of chick embryos in our laboratory, in which an antisense oligonucleotide that downregulates Cx43 was applied to one eye in ovo on embryonic day 2 and both eyes were analyzed 2 days later. When compared with untreated, sham-treated and sense-oligonucleotide-treated controls, the antisense-treated eyes were found to be significantly smaller, containing smaller but normally organized retinae with fewer mitotic figures and no evidence of increased cell death (6).

That experiment did not attempt to distinguish between the multiple roles now attributed to Cx43 protein in forming complete intercellular gap junctions and undocked (nonjunctional) hemichannels, in promoting homophilic intercellular adhesion, and in interacting with nexus cytoplasmic proteins. Experiments in vitro subsequently addressed the hemichannel issue by demonstrating a role for ATP, released through mimetic-peptide-sensitive hemichannels on the retinal face of the pigment epithelial layer, in shortening the M phase of neuroepithelial cell cycles (78). Taking this evidence together with early electron-microscopic reports and later tracer-coupling and calcium-imaging studies that provided unequivocal evidence of gap junctions (77), it seems that both gap junctions and hemichannels must coexist in the optic cup and may cooperate in this role. It has, however, been suggested that the ATP-releasing hemichannels might instead be constructed from pannexins, which form large-pore channels that may open more readily than connexin-based channels do under physiological conditions (117). Confusingly, the permeability of pannexin hemichannels can be acutely reduced by certain “connexin-specific” mimetic peptides, even though pannexins do not contain the amino-acid sequences to which such peptides are designed to bind (117).

Clear links have long been established in nonneuronal cells between brief elevations of intracellular free Ca$^{2+}$ (calcium transients) and entry to specific phases of the cell cycle, these transients being particularly prominent at the G1/S and G2/M transitions and the decision to exit the cycle (88). In the mammalian cortical plate, large clusters of neuroepithelial cells are gap-junctionally coupled, and their cells are synchronized in cycle phase. This has led to the proposal that coupling may allow spontaneous calcium transients to propagate, facilitating synchronized cycling (10, 73). In the retina, therefore, it has been suggested that ATP released into the extracellular space from pigment-epithelial hemichannels may act on neuroepithelial P2Y purinergic receptors to promote the observed calcium transients. These transients may then be reinforced by gap-junctional propagation, coordinately regulating the cell cycles of coupled cell clusters (78). The mechanisms linking

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**FIGURE 2.** Guide to the approximate timing of some events in retinal development

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P2Y receptor-activated calcium transients to the mitotic cycle in retinal neuroepithelial cells are currently unknown. However, calcium-imaging studies have been performed on primary cultures derived from adult Müller glia, the final progeny of these cells, and in these studies three mechanistic observations have been made: first, calcium ion influx from the extracellular space is necessary for increased proliferation; second, calcium-permeable channels and calcium-dependent potassium (BK) channels are required; and third, the duration of ATP-evoked calcium transients is directly correlated with the proliferation rate.

Most recently, an additional "proteomic nexus" role for Cx43 has been outlined, which may involve protein-binding domains in the carboxy-terminus cytoplasmic tail of the protein, controlling development of the optic cup independent of channel activity. Retinal pigment epithelial cells, which express Cx43 strongly, were transfected in vitro with either full-length Cx43 or a carboxy-terminus-truncated Cx43 mutant in which the channel-forming sequences were intact (56). Although this mutant was shown to support normal junctional communication, it was unable to induce either a rise in intracellular cAMP or the differentiation of pigment cells that normally accompanies such a rise under culture conditions in which full-length Cx43 was shown to have both actions. Pharmacological manipulations of cAMP were able to oppose and override the effects of transfection with either form of Cx43, confirming that the actions of cAMP were dependent on the standard pharmaceutic found to inhibit both Cx43 affecting either form, and comparable in vivo, if not to impact similar to the other organs.

The mechanism of the region of Cx43 regulates epithelial morphogenesis, not the nonretinal movement that it has been characterized to the nucleus effect, with which Cx43 cytoplasmic tails family tyrosine-carboxy-terminus, related to the n bilateral, it has at least two important regulation profiles. In the retina as mice, rats, but its later stage, rats have no nexins in retinal epithelia, established and the albinus mammalian characterized only as perinatal albinism as well as hemizygous. The retina is thicker than in mice an index that is followed by an eye retina down in differentiation (50); melanin and RNA are lacking in albinos due to highest, and from retina or extracellular (50) or inside, these differences are not only by proliferation but regulator of neurogenesis in adult retina. Perinatal albinism, niated by large amount, were returned at differentiation of either the same Cx43-sp at used to suppress...
Following development activity, express Cx43 or other full-uncoupled Cx43 sequences were found to suppress intercellular communication without affecting either cAMP levels or differentiation. Comparable effects on pigment epithelial differentiation in vivo, if they occur, would certainly be expected to impact strongly on neuroepithelial proliferation and organization (50). The mechanism by which the carboxy-terminus region of Cx43 regulates these processes in retinal pigment epithelial cells is unknown. However, studies of other tissues have not only confirmed the importance of connexin 43 in nonretinal models have led to the surprising observation that it has the ability to become selectively localized to the nucleus (22), much like β-catenin, the nuclear effector protein of the canonical Wnt pathway, with which Cx43 can also bind in complexes at the cytoplasmic side of the plasma membrane (31). Src-family tyrosine kinases, which also interact with the carboxy-terminus region of Cx43, can also be translocated to the nucleus (89); thus, although Cx43 itself lacks a conventional nuclear targeting sequence (22), it has at least two potential routes to the nucleus by association, and the potential to interfere in at least two important cytoplasmic signaling pathways that regulate proliferation.

In the retinae of mammals that are born blind, such as mice, rats, and ferrets, neurogenesis begins in utero, but its later stages can be studied postnatally. Studies of rats have not only confirmed the importance of connexins in regulating retinal proliferation but also established an interesting link from the pigment epithelium to the well-known retinal pathway defects of albino mammals. Tyrosinase-deficient albino rats are characterized by hyperproliferation of the neural retina as well as hypopigmentation of the pigment epithelium. The retina in albino is transiently up to 20% thicker than in pigmented littermates, with a mitotic index that is 50% greater at its peak; and this is followed by an excess of pyknotic cell death that thins the retina down later, modifying its spatiotemporal differentiation (50). Levels of DOPA (a precursor of both melanin and dopamine, another known cell-cycle regulator) are several-fold higher in pigmented eyes than in albino during the period when the mitotic index is highest, and the administration of L-DOPA (a precursor of both melanin and dopamine, another known cell-cycle regulator) is several-fold higher in pigmented eyes than in albino during the period when the mitotic index is highest, and the administration of L-DOPA to eyes in vitro (50) or in vivo (107) has been found to suppress these differences. A link to gap junctions was suspected, not only because of their known role in regulating proliferation but also because dopamine is a powerful regulator of gap-junctional communication in the adult retina. Subsequently, the high mitotic levels in perinatal albino retinae were found to be accompanied by large elevations in Cx43 protein, and both were returned toward normal by the in vivo administration of either L-DOPA (but not D-DOPA) or the same Cx43-specific antisense oligonucleotide that was used to suppress proliferation in the chick (106).

CAMP were downstream of Cx43. In contrast, standard pharmacological gap-junction blockers were found to inhibit intercellular communication without affecting either cAMP levels or differentiation. Comparable effects on pigment epithelial differentiation in vivo, if they occur, would certainly be expected to impact strongly on neuroepithelial proliferation and organization (50). The mechanism by which the carboxy-terminus region of Cx43 regulates these processes in retinal pigment epithelial cells is unknown. However, studies of other tissues have not only confirmed the importance of connexin 43 in nonretinal models have led to the surprising observation that it has the ability to become selectively localized to the nucleus (22), much like β-catenin, the nuclear effector protein of the canonical Wnt pathway, with which Cx43 can also bind in complexes at the cytoplasmic side of the plasma membrane (31). Src-family tyrosine kinases, which also interact with the carboxy-terminus region of Cx43, can also be translocated to the nucleus (89); thus, although Cx43 itself lacks a conventional nuclear targeting sequence (22), it has at least two potential routes to the nucleus by association, and the potential to interfere in at least two important cytoplasmic signaling pathways that regulate proliferation.

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Roles in Controlling Nuclear Migration and Neuronal Lamination

In the retinal neuroepithelium, as in other derivatives of the neural plate, the cell cycle is tightly linked to a dynamic set of oscillatory nuclear movements known as interkinetic nuclear migration (FIGURE 3). These movements give the neuroepithelial layer a pseudo-stratified structure in which mitosis occurs only among those nuclei that lie at the apical surface, abutting the pigment epithelium across the former ventricular space. Conversely, DNA replication occurs only among basal nuclei, lying closer to the lens. The benefits of interkinetic migration remain speculative, but it may have evolved to allow neuronal progenitors to make selective surface contact with others in the same cycle phase, while they are choosing their differentiated fates, without requiring the whole progenitor population to cycle in synchrony (70). Consistent with this, when interkinetic migration is disrupted by a motor-protein defect in the mikre oko (mok) zebrafish, Notch signaling between neighbors is weakened, and changes occur in cell proliferation and fate (27).

The underlying cytoskeletal and regulatory mechanisms of interkinetic migration are also poorly understood; a recent review notes many similarities to other forms of nuclear movement and also some critical differences (4). For example, the centrosome remains static, close to the ventricle, while the interphase nucleus migrates basally in G1 and returns apically in G2 (125), in the mammalian neocortex, at least, this nuclear movement is regulated from a considerable distance by a novel centrosomal protein, Cep130, that is preferentially expressed in neural progenitors (122). The extreme elongation of translocating nuclei during interkinetic migration (4, 79) has been interpreted as an effect of towing forces exerted on the nuclear membrane by cytoskeletal motors attached to the microtubular cytoskeleton, because these nuclei round up when the nucleus returns to the apical surface for mitosis. By contrast, in postmitotic migrating neurons throughout the CNS, the centrosome usually, although not always (111), migrates close to and ahead of the nucleus and has been proposed to transmit towing forces to it through a special, cage-like arrangement of perinuclear microtubules (98), which could be an adaptation for higher migration rates without nuclear rupture.

In interkinetic and postmitotic migration alike, however, nuclear motion is distinctly irregular and salatory (79, 98). In discussing neuroepithelial proliferation above, we suggested that calcium transients could be propagated by gap junctions into neighboring progenitor cells to coordinate their cell cycles (78). Evidence is also accumulating that the saliary nuclear migration of progenitors and young neurons may be controlled by calcium transients, which have previously been found necessary for the migration of human neuronal leukocytes and insect gial cells (65, 79).
The strong, but not unbreakable (4), interdependence of cycle phase and nuclear position that characterizes interkinetic migration is consistent with the notion that calcium transients may regulate both coordinately, as is the pattern of intercellular coupling (FIGURE 4).

To investigate the relationship between calcium transients, nuclear migration, and gap-junction proteins, we used time-lapse confocal microscopy to study chick retinae on embryonic day 5, when the first wave of postmitotic neurons is beginning to leave the ventricular surface but only the nuclei of cycling neuroepithelial progenitors are moving toward it (79).

Three in every four saltatory nuclear movements (whether directed toward the ventricle or away) directly followed detectable calcium transients, and many of the transient/movement pairings were synchronized among neighboring cells. Pre-incubation with BAPTA to buffer the transients greatly retarded the movements, as did several treatments that are known to reduce gap-junctional coupling among cycling neighbors: these included transfection with a dominant-negative Cx43, application of a Cx43-specific antisense oligonucleotide, and application of any one of three pharmacological gap-junction blockers. A hemichannel-blocking mimetic peptide also reduced movement, but to a lesser extent, consistent with an inhibitory effect on gap-junctional assembly (79).

Detailed studies of migrating cerebellar granule neurons have shown their overall rates of movement in slice cultures to be correlated with the amplitude and frequency of calcium transients and reduced by treatments that suppress them (77). More recent work has shown that the loss of these calcium transients directly regulates the cessation of granule-cell migration (59). Our observations imply that interkinetic and postmitotic nuclear movements in the retina have similar dependencies.

If the intensum transients both progenitors and their direct progenitors, as in cerebellar granules control the direction of nuclear movement is marked by a polarity complex. The interkinetic migration process (at least in the retina) is induced by a direction of nuclear migration is marked by an apical polarity complex. The interaction between these phase changes is mediated by the cytokeletal complexes. However, we have evidence that gap-junction proteins/guides disrupt communication as the cycle, and interkinetic movements are disrupted as the cells begin to leave the ventricular zone. Our observations indicate that gap-junction proteins/guides are critical (33). To investigate the relationship between calcium transients, nuclear migration, and gap-junction proteins, we used time-lapse confocal microscopy to study chick retinae on embryonic day 5, when the first wave of postmitotic neurons is beginning to leave the ventricular surface but only the nuclei of cycling neuroepithelial progenitors are moving toward it (79).

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If the intensity, frequency, and/or duration of calcium transients regulate the rate of nuclear movement in both progenitors and postmitotic neurons, what controls its direction? In migrating olfactory neuronal progenitors, as in postmitotic hippocampal neurons and cerebellar granule neurons, extracellular guidance cues control the extension of a leading process that is marked by association with the centrosome and stabilized by the evolutionarily conserved Par-aPKC polarity complex. The nucleus always migrates into this process (at least in vitro), and, if a new process is induced by a manipulation of extracellular cues, direction of nuclear migration changes only after the polarity complex has repositioned the cell (46). In cycling neuroepithelial cells, by contrast, the regular reversals of nuclear migration between the G1 and G2 phases plainly do not involve cell repolarization, because apical features such as specialized membrane proteins, junctional adhesion complexes, and polarity complex components persist throughout interphase. However, we have recently obtained preliminary evidence that gap-junction proteins play some part in these phase-change-related reversals. When agents that disrupt Cx43 expression or gap-junctional communication are applied over large fractions of the cell cycle, nuclei accumulate at both ends of the normal interkinetic migratory range, and some of the affected cells begin to differentiate ectopically, disrupting the normal pattern of retinal lamination (7). Whether these observations primarily reflect disruptions of migration or cell-cycle progression is not yet known.

We end this section with a necessarily brief outline of two studies that adduce nonjunctional roles for Cx43 in the migration of postmitotic neurons outside the retina. In embryonic rodent neocortex, either the acute knockdown of Cx43 by electroporation of a specific shRNA in utero (35) or its conditional knockout in neuroepithelial cells and radial glia, driven by a nestin promoter (16), both demonstrate such roles. In the first study, co-electroporation of a series of closed-channel or carboxy-terminus-truncated mutant connexins along with the shRNA, to rescue different aspects of Cx43 function, showed that channel conductance and carboxy-terminus binding were dispensable for migration but that junctional adhesion was critical (33). In that study, although the shRNA affected both neurons and their neuroepithelial precursors/guides, the interpretative focus was on the neurons because the migration deficit was found to persist when GFP-tagged, shRNA-electroporated neurons were combined with normal neuroepithelial cells, either in vivo or in vitro, whereas untagged neurons appeared to migrate normally, even in electroporated regions. In the second study, focusing on nestin-expressing, Cx43-deficient neuroepithelial cells, rescue of neuronal migration was achieved by the transfection of wild-type Cx43 but not a carboxy-terminus-truncated version, thereby implicating the cytoplasmic tail of the protein. Consistent with this, mice homozygous for a carboxy-terminus truncation that would not be expected to alter either conductance or adhesion were found to show similar migration defects to the conditional Cx43 knockout mice (16).

Although the adhesive extracellular-loop domains of Cx43 may be critical at the neuronal face of each plaque, its cytoplasmic binding properties may dominate its role at the neuroepithelial face.

These two studies, although superficially contradictory, may represent opposite faces of a single coin, their common currency being the actin cytoskeleton. This is extensively remodeled around all focal adhesion sites, including gap-junctional plaques in migrating neurons (35), and it interacts with the carboxy-terminus tail through a rich set of nexus proteins, including drebrin and ZO-1 (16, 31). Although the adhesive extracellular-loop domains of Cx43 may be critical at the neuronal face of each plaque, its cytoplasmic binding properties may dominate its role at the neuroepithelial face.

Roles in Controlling Programmed Cell Death

Programmed cell death is a major feature of retinal development in all vertebrates. Its first, postneurogenic, phase appears to depend mainly on the supply of dopamine, neurotrophins, and insulin-related factors, whereas its later, circuit-refining phases probably also involve excitatory neurotransmission at NMDA receptors (63, 115). Gap-junction proteins (both connexins and pannexins) have critical and complex roles in regulating, spreading, and effecting cell death in many pathological contexts (for reviews, see Refs. 26, 81, 86, 105), including localized retinal injury (101), so there is a case for considering such roles in physiological contexts too. Indeed, dopamine, which reduces apoptotic death in newborn rat retinae (114), is also a potent modulator of gap-junctional communication in adult retinae, and the rate of apoptosis in developing retinal explants can be modulated by the unspecific gap-junction blocker, octanol (63), although this effect may be secondary to effects on proliferation or migration, as discussed above.

In the most detailed study of this role to date (20), freshly isolated rodent retinae were scrape-loaded with cytochrome C—a gap-junction-impermeant
apoptotic agent) mixed with rhodamine-dextran (also impermeant) and Neurobiotin (a gap-junction-permeant tracer) to determine the relationship between junctional coupling and cell death. Unreated control retinae were then compared with retinae pretreated in vivo by systemic injection of the gap-junction blocker carbenoxolone. In retinae from untreated mice, TUNEL-positive pycnotic cells in the inner retinal layers were found to be significantly clustered, consistent with the transmission of cell death signals between close neighbors, perhaps through gap junctions. In retinae from mice pretreated with carbenoxolone at the same age (postnatal day 5), fewer tracer-coupled cells were seen after scrape-loading, and dying cells were significantly less clustered. To test more directly for a causal link between coupling and death, injections of another gap-junction-permeant tracer, Alexa Fluor 488, with or without cytochrome C, were made into single cells in older (postnatal day 13) rat retinae in vitro. Whereas cytochrome C did not directly affect cells, the incidence of both coupling and death being reduced by carbenoxolone. Taken together, these findings imply that patterns of gap-junctional coupling may directly influence the spatial distribution of cell death in the developing retina and perhaps also its overall incidence.

There may also be scope for seeking out developmental equivalents of other known pathological roles for gap-junction proteins, including nonchannel roles. In cardiac myocytes, for example, Cx43 has been localized by several techniques to the membranes of cells, the incidence of both coupling and death being reduced by carbenoxolone. Taken together, these findings imply that patterns of gap-junctional coupling may directly influence the spatial distribution of cell death in the developing retina and perhaps also its overall incidence.

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Roles in Synaptogenesis and Circuit Formation

We have noted that many signaling modes interact in the embryo, and this is especially evident when neuronal circuits are being established. Axonal growth cones must read and integrate multiple extracellular signals even before synaptogenesis occurs (12). Distinct patterns of spontaneous or evoked neural activity, in which gap junctions play a part, are then used to refine synaptic patterns as development proceeds (12).

In the earliest stages of differentiation, the neurotransmitters GABA (γ-aminobutyric acid) and glycine have depolarizing, excitatory actions on postsynaptic neurons, becoming inhibitory only when those neurons express the KCC2 ion co-transporter (8). In both mouse and turtle retina, this maturation shift from excitation to inhibition occurs only after the stratification of synapse-bearing amacrine cell processes (62, 124). In electrical terms, therefore, the earliest neurochemical synapses are synergistic with gap junctions and perhaps functionally interchangeable with them. Indeed, in the snail Helisoma, a dynamic negative interaction between regenerating electrical and chemical synapses has been directly demonstrated: cholinergetic axons were found to inhibit gap-junctional coupling when accelerated by several techniques to the membranes of cardiac muscle remains an open question because there seem to have been no appropriate studies. Whether connexins have a recognized role in preconditioning the heart to resist ischemia, independent of gap-junctional coupling, was found to accelerate the reestablishment of chemical transmission (194). Evidence for a similar interaction has also been reported after blockade of chemical transmission in developing mammalian motoneurons (68).

Similar phenomena have been observed, although indirectly, in the vertebrate retina. We have already mentioned the spontaneous, locally synchronized elevations of intracellular calcium that occur in the previusal retinae of reptiles (93), birds (13), and mammals (120). These calcium transients are able to propagate intermittently, slowly, and in random directions as waves across the plane of the retina, in association with bursts of action potentials in retinal ganglion cells (14). The slowness of their propagation allows retinal ganglion cells to embed detailed information about their relative retinal positions into the correlative structure of their firing patterns. Retinorecipient central target nuclei then exploit this “free” epigenetic information in generating eye-specific patterns of laminar segregation and refining the spatial order of first- and second-order retinotopic visual maps (11, 49, 108). The basis for wave propagation has been contentiously variously by gap-junctional coupling and neurochemical (excitatory GABAergic, acetylcholinergic and/or glutamatergic) transmission, and there is still controversy about the role of each (109). There is broad agreement, however, that the earliest waves in birds and mammals precede synaptogenesis and are susceptible to drugs that block gap junctions (13, 103, 121), whereas waves at later stages resist gap-junction blockers but succumb to drugs that block nicotinic cholinergic and/or glutamatergic transmission (108).

Interestingly, there is evidence to suggest that electrical and neurochemical synapses in the developing retina show a complementarity surprisingly like that observed in Helisoma. In a mouse mutant lacking choline acetyltransferase, and thus lacking acetylcholine, retinal waves were found to reappear a few days after birth but were not generated by the mechanism normally in use at that time: they were susceptible to gap-junction blockers rather than to glutamatergic antagonists. A similar homeostatic upregulation of gap-junction-dependent waves was observed in vivo in retinal ganglion cells, when normal embryonic nicotinic receptors were replaced by a gap-junction-permeant tracer, and waves became to a gap-junction blocker. In developing mammalian retinae, this task is also accomplished by carbenoxolone. In retinae from mice pretreated with carbenoxolone at postnatal day 13, fewer tracer-coupled cells were seen after scrape-loading, and dying cells were significantly less clustered. To test more directly for a causal link between coupling and death, injections of another gap-junction-permeant tracer, Alexa Fluor 488, with or without cytochrome C, were made into single cells in older (postnatal day 13) rat retinae in vitro. Whereas cytochrome C did not directly affect cells, the incidence of both coupling and death being reduced by carbenoxolone. Taken together, these findings imply that patterns of gap-junctional coupling may directly influence the spatial distribution of cell death in the developing retina and perhaps also its overall incidence.

There may also be scope for seeking out developmental equivalents of other known pathological roles for gap-junction proteins, including nonchannel roles. In cardiac myocytes, for example, Cx43 has been localized by several techniques to the membranes of cells, the incidence of both coupling and death being reduced by carbenoxolone. Taken together, these findings imply that patterns of gap-junctional coupling may directly influence the spatial distribution of cell death in the developing retina and perhaps also its overall incidence.
observed in wild-type mice after just a few hours of nicotinic receptor blockade (100). Similarly, two mutant mouse lines lacking the β2 subunit of the nicotinic acetylcholine receptor have also been reported to revert to a gap-junctional mechanism for generating retinal waves, becoming sensitive to a relatively selective gap-junction blocker, 18β-glycyrrhetinic acid, at stages when normal mice have lost this sensitivity (102).

It is tempting to speculate on a possible role for the extracellular proteoglycan agrin in this homeostatic process, because agrin has been shown to control a range of gap-junction-mediated electrical coupling to nicotinic cholinergic transmission within the rat adrenal medulla (87), and agrin is also apparently ubiquitous at retinal synapses (9, 58). Perhaps the various disruptions to cholinergic mechanisms lead to agrin dispersal, allowing electrical transmission to be restored. However, it is not certain that these experiments demonstrate a reversion to true electrical transmission, because a reversion to extrasynaptic neurochemical signaling is also a possible cause. Adenosine signaling is also known to play a part in the early stages of retinal wave generation (100), and these experiments did not rule out a role for undocked gap-junction hemichannels, which might be involved in the release of either adenosine or nucleotides such as ATP that are readily cleaved to adenosine by ectonucleotidases.

Thus far we have considered synaptogenesis and circuit formation as if they were independent of the processes controlling cell number and fate. However, the maturational shifts that underlie the emergence of inhibitory transmission and neurochemical wave propagation in the inner retina may also have implications for proliferation in the outer retina, where bipolar cells and rod photoreceptors, as well as Müller glia, are still being born. In a detailed study of the perinatal rabbit retina, Syed et al. (103) were able to combine multi-electrode-array analyses of ganglion-cell firing, not only in mammals, where it is found in cones (61) and OFF-cone-bipolar cells (36), but also in the cone-dominated chick retina (55). Although Cx36 appears not to be expressed in rods (23), 18β-glycyrrhetinic acid, at stages when normal mice have lost this sensitivity (102).

Roles that Persist into Adulthood

We have focused, so far, on roles of gap-junction proteins that end when the developing retina reaches maturity. However, gap-junction proteins also have crucial functions in the mature retina, especially in controlling feedback to cone photoreceptors through the horizontal cell arrays (72) and in the circuitry of low-light vision (24), where they contribute to signal averaging and noise reduction (48, 96). Coupling may have metabolic consequences too. For example, in the adult rat, glycine can be found in cone bipolar cells that lack its selective transporter Glyt-1, having entered these cells through gap junctions from amacrine cells that do express the transporter. These junctions become functional only in the second postnatal week (85).

Space precludes a detailed discussion of these adult roles, but their emergence is undeniably part of retinal development. Since our last review (17), the main frontier of advance has been the identification of new connexin and pannexin gene family members and the locations and roles of their encoded proteins. This has been complicated by the diversity of the species in which these proteins have been studied, the extent of gene duplication and loss during phylogeny, and the dominance of a nomenclature for connexins that is based on predicted molecular masses and has no simple relationship to orthology (18). However, at least four connexins (Cx36, Cx45, Cx50, and Cx57/62) are known to be expressed selectively by different classes of adult retinal neurons in mammals (72), supplemented by Cx26 in fish and turtle horizontal cells (83), by Cx43 in Müller glia and astrocytes, and by Cx37 in vascular endothelial cells (97). The main connexin of each neuronal type has usually been confirmed only in the adult (and often in only one species), but in some cases the time of emergence of its characteristic coupling pattern was already known. Cx36 has been associated with photoreceptor coupling, not only in mammals, where it is found in cones (61) and OFF-cone-bipolar cells (36), but also in the cone-dominated chick retina (55). Although Cx36 appears not to be expressed in rods (23, 61), it is known to be essential for the processing of rod signals in the inner retina, where it has been localized to All amacrine/bipolar gap junctions (24). The timing of its appearance in photoreceptors has not, however, been reported separately from its upregulation in inner retinal neurons, which takes place during the first 2 postnatal weeks in mice (41). One of the roles of Cx36 in the inner retina is to couple alpha ganglion cells to each other and to amacrine cells (91), and the...
emergence of alpha ganglion cell coupling has been followed through the first 3 postnatal weeks by tracer injection in ferrets (80). Cx43 is expressed in some types of cone bipolar cell, some types of amacrine cell, and at least one subset of ganglion cell types: the bistriated, direction-selective ganglion cells (92). In the rabbit, DeBoer and Vaney (25) found that these cells gradually decouple from their overlapping neighbors during the 10 days after birth, forming four independent, minimally overlapping mosaic arrays, only one of which remains tracer-coupled. They suggested that the changing pattern of these cells may be linked to their type-specific synaptic differentiation, which would be consistent with ideas explored in the previous section.

Cx26 is found in type A horizontal cells, whereas type B horizontal cells in rabbits and the single B-like horizontal cell in mice both contain Cx57 (72), to which Cx62 is orthogonal in a range of other mammals from opossum to human (18). Mature patterns of type-specific coupling have previously been found in neonatal rabbits by tracer and dye injection into horizontal cells of both types (51), so the development of these patterns is clearly prenatal. In the chick, Cx50 is expressed only weakly during the period of horizontal cell generation and migration but more strongly after hatching (54).

Finally, undocked hemichannels have been proposed to lie deep inside the synaptic terminals between horizontal cells and cones in the adult retina of teleost fish and reptiles, where they are thought to deliver current into the synaptic cleft in an ephaptic, focal, modulating role that may underpin horizontal cell feedback onto the cone array. RT-PCR and immunocytochemical studies have suggested that this role might exploit the properties of Cx26, a naturally carboxy-terminal-truncated connexin with an unusual pattern of voltage gating (52, 83). More recently, evidence has also been reported for the involvement of a pannexin-based hemichannel in this role in a teleost fish (84). Although nothing more is known, at present, it will be very interesting to discover whether it is unique to the retina.

so it is also having to abandon the notion that each protein has just one cellular role. We have outlined ways in which a protein such as Cx43 can regulate intercellular communication channels, externally exposed hemichannels, intercellular adhesion mechanisms, and cytoskeletal linkages, and we have discussed evidence from the developing retina that connexins coordinate the cell cycle, cell culture migration and cell survival, mediate synaptogenesis, and then take on key physiological roles in the adult. Thus, the roles of at least one connexin may extend beyond the plasma membrane to the cytoplasmic vesicles into the nucleus and even the mitochondria, and beyond protein interactions into the control of gene expression. The nexus, in all its guises, may still have much more to communicate.

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**References**


can regulate adhesion mechanisms and have the ability to retain normal cell migration, growth, and the adult. Thus, extend beyond vesicles into adhesion molecules, and beyond gene expression we have much more.

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