Nonredundant Gap Junction Functions

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The need for molecular heterogeneity of gap junction channel proteins in vivo has been enigmatic. Recently, functional replacement of one channel gene with another in mice and flies has revealed that cellular health depends not simply on gap junction communication but also requires the correct type of intercellular channel subunit.

Gap junctions allow the cell-to-cell diffusion of ions and signaling molecules between the cytoplasm of neighboring cells in a variety of tissues and cell types (3, 6). Junctional coupling is present in all multicellular animals, yet unrelated gene families fulfill this function in vertebrate and invertebrate organisms (Fig. 1, A and B). In humans, there are at least 20 connexin (Cx) genes (6), whereas flies and nematodes use 8 and 25 innexin (Inx) genes, respectively (9). Although the number of fly genes is smaller, this organism increases functional diversity by using alternative splicing to produce several distinct intercellular channel proteins from a single gene (9). It remains unclear why such a large number of gap junction proteins is required to provide this universal type of intercellular communication, which is used by virtually all cells that are in direct contact.

Gap junction channels formed by different subunits have distinctive permeability, characteristic voltage gating, and specifically regulated assembly; thus intercellular communication mediated by individual gap junction genes should have diverse effects on tissue function. Data demonstrating that each connexin displays unique functional properties has been obtained from a variety of in vitro assays (8). Less data is available for the innexins, owing to their more recent discovery, but they do form channels in expression systems (9) and their macroscopic gating behaviors are similar to the connexins (Fig. 1, C and D). Although more in vitro work remains to be done with the innexins, a landmark comparison of invertebrate and vertebrate channel behavior (2) suggests that the same range of functional properties seen in the connexin family will eventually be demonstrated. Relating the channel diversity observed in vitro to tissue function in vivo has remained a major challenge.

Evidence for wide-ranging, gap junction-dependent processes in vivo has emerged from studies of connexin knockout mice and human diseases caused by connexin mutations (6, 18), as well as analysis of innexin mutants in nematodes and flies (9). En masse, these data provide overwhelming support for the importance of junctional coupling in many physiological processes, but because they rely on the selective loss or mutation of a single gene they fail to directly address the need for subunit diversity. Both innexin and connexin genes are expressed in complex and overlapping patterns, so that most cell types have more than one intercellular channel subunit (6, 14). The phenotypes resulting from gene mutation or deletion could consequently result from either a partial reduction in total cellular coupling or the loss of a specific functional activity provided by the targeted gap junction gene. Recent studies have addressed this issue by functionally replacing gap junction subunits rather than deleting them, thus maintaining the magnitude of gap junction coupling while reducing subunit diversity. These studies (4, 10, 16) clearly establish that intercellular communication is finely tuned to the physiological needs of a specific tissue and that the diverse gap junction channels providing it are not functionally redundant.

**Cx43 replacement produces a fate worse than death**

Cx43 is widely expressed in many tissues, including the heart and testis. Therefore, the observation that Cx43 knockout mice died at birth from cardiac malformation was not completely unexpected (11). This result raised the simple question of whether another connexin could substitute for Cx43 and restore viability. Functional replacement of Cx43 with Cx40 by genetic knock-in produced animals (referred to as Cx43KI40) that did indeed survive beyond birth (10). The cardiac malformation defect of Cx43-deficient mice was corrected by replacement of Cx43 with Cx40, and the hearts of Cx43KI40 animals appeared to develop and function normally. However, the dramatic rescue from certain death also produced a new and unexpected spectrum of gap junction-dependent defects.

For example, although the Cx43KI40 mice were viable, male knock-in mice were sterile and had severely hypotrophic testicles that lacked sperm (Fig. 2). Spermatogenesis normally proceeds according to an orderly pattern along the seminiferous tubules of the testis. Germ cells are interspersed within tubular supporting Sertoli cells that participate in the nutrition and protection of spermatogonia. It has been shown that seminiferous tubules have multiple routes of gap junction communication that differ in both permeation and gating (12). This functional diversity permits regulated communication among the Sertoli cells and many germ cell stages within the seminiferous epithelium, which in turn is thought to be important for the initiation and maintenance of spermatogenesis. Besides the obvious testicular defect, the knock-in mice exhibited additional problems that were not as clearly defined. For instance, many homozygous Cx43KI40 animals never made it to adulthood, ~40% of the pups died during the first few postnatal weeks, and the surviving animals were notably under-
weight (10). Interpretation of these observations was complicated by the knock-in animals’ generally poor health that could have resulted from Cx43 substitution in multiple organs. In summary, replacement of Cx43 with Cx40 restored basic cardiac function and life but produced poor health and reproductive failure, a fate perhaps even worse than death.

It is difficult to speculate on the molecular mechanisms underlying this outcome, although there are clear differences in intrinsic properties between Cx43 and Cx40. Gap junction communication can be simply divided into two broad categories: ionic coupling mediating the intercellular passage of small ions such as potassium and biochemical coupling facilitating the sharing of small molecules like cyclic nucleotides between communicating cells. Unitary conductance of Cx43 channels is about half that of Cx40 channels; thus Cx40 would result in an increase in ionic coupling between two cells joined by the same number of channels. In contrast, Cx43 channels are about five times more permeable to fluorescent dyes of similar size and charge as many second messengers than are Cx40 channels; thus Cx40 would produce less-efficient propagation of biochemical signals between two cells joined by an equivalent number of channels (15). Therefore, the physiologically important difference between these two connexin channels with regard to testicular development is

FIGURE 1. Unrelated gene families with similar functional properties encode gap junctions in invertebrates and vertebrates. A: schematic illustration of identity relationships between Drosophila innexin amino acid sequences. Two grasshopper (Sa, Schistocerca americana) and two nematode (Ce, Caenorhabditis elegans) innexins were included for comparison. The Drosophila shakB gene is alternatively spliced to generate at least 3 unique gap junction proteins. The nematode innexins group outside the insect innexins, suggesting that insect and nematode gap junction genes arose from a common ancestor early in evolution. B: dendrogram of human connexin protein sequences. Connexin genes are distributed into 3 subgroups recognized by the Human Genome Nomenclature Committee (i.e., GJA1, GJB1, and GJC1). Connexins display high homology across vertebrate species and have no identity with innexins. C and D: voltage gating of innexin and connexin channels. The effect of transjunctional voltage on the gap junction currents developed by pairs of cells expressing an innexin (C, Ce Inx-3) or a connexin (D, chicken Cx45.6) is shown. Depolarizing and hyperpolarizing voltage steps of 10, 30, 50, and 70 mV were applied to 1 cell of each pair. At transjunctional voltages >20 mV, innexin and connexin junctional currents slowly decreased over the time of the voltage step with similar kinetics. In both cases, the voltage-dependent closure was not complete. The voltage gating of the innexin channel fell well within the range of behaviors exhibited by members of the connexin family (reviewed in Ref. 8). Adapted from Refs. 14, 18, and 19.
Clarity was maintained, and little if any intragranular precipitate was observed. Microscopy examination revealed that the nuclei were of normal size and structure, but the intranuclear opacity was evident (Fig. 3C, D). The eyes and lenses of knock-in animals were 25% and 34% smaller, respectively (16). This demonstrated that functional replacement of Cx50 with Cx46 was not able to correct the growth deficit that resulted following simple knockout of the Cx50 gene. Despite the fact that the Cx50KI46 lenses displayed a growth defect, they were able to achieve and maintain the proper state of transparency (Fig. 3). Lenses dissected from Cx50KI46 eyes lacked the nuclear cataracts exhibited by Cx50 knockout lenses (16). Thus functional replacement of Cx50 by Cx46 prevented the loss of crystallin solubility and cataracts that occurred following knockout of the Cx50 gene.

Since the lens is a simple organ requiring the function of two distinct connexin genes for proper growth and differentiation, it is an ideal model to test hypotheses about gap junction subunit redundancy. It was surprising that the targeted deletion of either Cx46 or Cx50 resulted in significantly different phenotypes, suggesting that these outcomes were influenced by more than simple reductions in the total amount of communication, which would be expected to occur in either knockout. In the Cx50KI46 mice, reduced lens growth led to microphthalmia, very similar to the growth defect resulting from Cx50 deletion, whereas lens fibers were able to maintain homeostasis and avoid crystalline precipitation and cataracts that resulted following knockout of either Cx46 or Cx50. Thus the lens was able to segregate the contributions of gap junction communication to the control of normal growth and the maintenance of clarity. Clearly, Cx50 plays an indispensable role in normal lens and eye growth, but Cx46 alone cannot prevent lens cataract.

**Lack of innexin redundancy in *Drosophila* eye development**

Genetic knock-in by homologous recombination is not applicable to *Drosophila*, but many other genetic tools make it a good model. The issue of connexin diversity vs. quantity can be more precisely analyzed by exchanging connexins with a more restricted pattern of tissue distribution. One excellent model organ is the lens, which relies heavily on gap junction communication for cellular homeostasis. Two connexins, Cx46 and Cx50, are predominantly expressed in the lens, and mutations in either gene in humans lead to cataracts (18). In mice, knockout of Cx50 resulted in significantly reduced lens and eye growth in addition to mild nuclear cataracts (17). In contrast, deletion of Cx46 produced severe cataracts resulting from the failure to maintain crystallin solubility but did not alter ocular growth (7). These divergent phenotypes could have resulted from either different functional properties of the two channel types or their differential contributions to the magnitude and spatial arrangement of intercellular communication (1, 13).

To address these issues, a reduction in lens connexin diversity, but not channel number, was engineered in mice by genetic replacement of the Cx50 coding region with that of Cx46 (Cx50KI46). Cx50KI46 knock-in mice exhibited no differences in total body weight compared with wild-type; however, the eyes and lenses of knock-in animals were 25% and 34% smaller, respectively (16). This demonstrated that functional replacement of Cx50 with Cx46 was not able to correct the growth deficit that resulted following simple knockout of the Cx50 gene. The fact that the Cx50KI46 lenses displayed a growth defect, they were able to achieve and maintain the proper state of transparency (Fig. 3). Lenses dissected

**FIGURE 2.** Spermatogenesis absolutely requires Cx43. A: hematoxylin and eosin-stained sections of adult wild-type testes showed seminiferous tubules with large numbers of spermatocytes and mature spermatoza. B: in Cx43KI40 males, where Cx43 was functionally replaced with Cx40, no differentiating spermatocytes or mature sperm cells were found within the seminiferous tubules; only Sertoli cells remained. Thus simple junctional coupling provided by Cx40 could not rescue spermatogenesis in the absence of Cx43 and a connexin-specific mode of intercellular communication was required. L, seminiferous tubule lumen. Reproduced with permission from Elsevier Science from Ref. 10.

**FIGURE 3.** Lens growth and clarity depend on different connexins. A: adult wild-type lenses had no light-scattering opacities. B: Cx46 knockout lenses were of normal size but exhibited dense cataracts. C: Cx50 knockout lenses were smaller than normal and contained a fine particulate precipitate confined to the nucleus. D: in Cx50KI46 lenses, where Cx50 was functionally replaced with Cx46, the cataract defect was rescued but the growth deficiency remained. Therefore, the lens requires Cx50 for proper growth and Cx46 to prevent cataract. Adapted from Refs. 7, 16, and 17.
flies an equally powerful system in which to address issues related to gap junction protein diversity. In one recent example, it was shown that innexins were not interchangeable in the development of neural function in the fly visual system (4).

In the *Drosophila* eye, photoreceptors located in the ommatidia send their axons to ganglion cells in the optic lobe of the brain. In adult flies, these two neuronal cell types do not communicate through gap junction channels but use instead chemical synapses. However, during development, temporary gap junctions are needed for the adult synapses to properly form. Specifically, the innexin genes *shakB* and *ogre* are required for the development of normal synaptic transmission between photoreceptors R1–G10 and their lamina ganglion cell partners in the adult. Mutations in either gene reduce neural transmission from the retina to the lamina (Fig. 4).

Transgenic expression of *ogre* in retinal neurons and *shakB* in lamina neurons during development rescued adult neural function (5). To test for specificity of innexin function in fly eye development, the sites of transgenic expression were switched. *Ogre* was expressed in the lamina neurons and tested for its ability to rescue *shakB* mutants, whereas *shakB* was targeted to photoreceptors in the *ogre* mutant flies. In both cases, electroretinograms (ERGs) showed that the inappropriate expression of genes could not restore normal synaptic transmission (Fig. 4). ERGs recorded the presynaptic photoreceptor potential and the postsynaptic response of the lamina ganglion cells and have three important features. The depolarization of the photoreceptor cells, lasting for the duration of the light pulse, is the main downward deflection. Additionally, there are upward on-transients and a downward off-transient reflecting the lamina ganglion cells’ responses to synaptic transmission from the photoreceptors at the beginning and end of the light stimulus. In *ogre* mutants, both transients were eliminated, whereas in *shakB* mutants the transients were reduced 50–75% in magnitude (4). Appropriate expression of *shakB* and *ogre* did rescue function, and a series of chimeras constructed from these two innexins revealed that sequences from the amino-terminal half of the proteins determined the specificity of the rescue (Fig. 4B). Thus, like the connexins in vertebrates, the highly homologous innexins were not interchangeable in their role to promote normal development and visual synaptic transmission.

**Summary**

Cell biology textbooks have historically described gap junction channels like simple pipes connecting cells, as though they were aqueous cylinders with a large diameter and were freely permeable to any solute <1,200 Da. Part of this misconception was due to the lack of clear ionic selectivity shown by other channel families. However, experiments carefully examining the movement of ions and dyes between cells expressing different connexins have revealed that there are connexin-dependent differences in permeation and that gap junction channels with different subunit composition can even discriminate between potent signaling molecules like cAMP and cGMP (8). Because most cell types express more than one connexin (or innexin), the permeability data suggest that loss of a single gap junction subunit within a given tissue (i.e., by genetic mutation) would not only change the macroscopic levels of ionic communication but would also significantly alter the range of molecules being exchanged between the coupled cells. Thus gap junction channels can no longer be considered passive conduits for the movement of mole-
cules with a molecular mass of <1,200 Da and must now be considered to provide a selective signaling route whose properties are determined by the molecular identity of the connexins or innexins available to the cells in direct communication.

This view has been validated by the genetic replacement experiments described in this review. Gap junction channel subunits have been shaped by evolution to fill precise physiological roles in tissues with diverse needs. Although simply providing nonselective ionic coupling may help explain normal cardiac function in the Cx43KI40 mice, the outright failure of spermatogenesis cannot be so easily clarified. Interestingly, knock-in of Cx32 for Cx43 produced a similar testicular defect with nearly normal heart function (10), further emphasizing the difference between ionic and biochemical modes of gap junction communication. In the analogous example of lens development, both Cx46 and Cx50 are highly permeable to monovalent ions but only Cx50 can direct normal lens growth, as though ionic coupling may be sufficient to provide clarity but more selective biochemical coupling regulates growth. Additional features are also likely to be important, as illustrated in the lack of mutual rescue of visual synaptic transmission between the ogre and shakB phenotype in flies (4), implying that other aspects of channel function such as assembly or gating were important. Together, these studies illustrate that different gap junction channel subunits are not functionally redundant in vivo. Thus wholesale exchange of small molecules <1,200 Da is no longer a sufficient working paradigm for gap junction channels; rather, they provide a precise molecular exchange tailored to suit the unique physiological needs of different cell types.

I thank Drs. Peter Brink and Richard Mathias for helpful discussions of the manuscript.

Work in my laboratory has been funded by National Institutes of Health grants EY-13163, AR-47102, and DC-05491.

References


